The Journal of Laboratory and Clinical Medicine

VOL. 50 AUGUST, 1957 No. 2

CLINICAL AND EXPERIMENTAL

A HEMODYNAMIC STUDY OF ATRIAL SEPTAL DEFECT AND ASSOCIATED ANOMALIES INVOLVING THE ATRIAL SEPTUM

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REVIEW OF THE LITERATURE

ATRIAL septal defect is one of the commonest congenital anomalies of the heart and one of those more recently amenable to surgical correction. Consequently, in recent years attention has been focused on the hemodynamic characteristics of the anomaly in an effort to learn more of the effect of this defect on the patient, to increase the accuracy of diagnosis, and to provide a more intelligent basis for the selection of candidates for surgical correction.

In uncomplicated atrial septal defects the shunt usually is left to right, and less commonly a right-to-left shunt of significant magnitude coexists.[1, 2]

Two of us (Swan and Wood) and associates[3, 4] have demonstrated that in the usual case of atrial septal defect the blood shunted from left to right is composed predominantly of blood draining from the right lung. Minimal right-to-left shunting is not uncommon and occurs preferentially from the inferior vena cava[5]; the right-to-left component is usually so small that the oxygen saturation of blood in the systemic arteries is within the range of normal and the patient has no cyanosis.

Abridgment of thesis submitted by Dr. Weidman to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Pediatrics.

Received for publication Jan. 14, 1957.

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The presence of a predominant right-to-left shunt with accompanying cyanosis has been demonstrated only in those patients with atrial septal defects complicated by pulmonary hypertension, cardiac failure, or associated congenital defect.

Because of the left-to-right shunt, the pulmonary blood flow is markedly increased in a patient with uncomplicated atrial septal defect, usually 2 to 3 times the systemic blood flow.[6, 7] The systemic flow has been reported normal or decreased.[6, 8] The pulmonary artery pressure is usually normal or slightly increased.[9] Pulmonary hypertension is relatively uncommon; only 4 of Limón's 50 patients with atrial septal defect had severe pulmonary hypertension.[2] If pulmonary hypertension does occur, it usually does not become evident until early adult life.[2, 10]

PURPOSE

This report is concerned with the basic hemodynamic data in a sufficiently large series of cases to permit substantiation of certain general conclusions with regard to patients with atrial septal defects. Additional features of these lesions, recently unrecognized, are considered in more detail.

Recent surgical progress in the correction of atrial septal defect[11] has necessitated comprehensive classifications[12, 13] of the variation of interatrial communication, for technical methods for the closure of such defects may differ according to the lesion present.

CLASSIFICATION OF INTERATRIAL COMMUNICATION

The following is a classification of interatrial communications that is of practical value.

I. Atrial septal defect
   - A. Foramen ovale type
   - B. Located cephalad to fossa ovalis and associated with anomalous connections of right pulmonary veins

II. Persistent common atrioventricular canal
   - A. Partial
   - B. Complete

III. Unusual communications via coronary sinus

IV. Valve-competent foramen ovale

A defect of the valve-competent foramen ovale type (IV) exists in about 25 per cent of normal human beings[12] and does not cause a detectable abnormality unless pressure in the right atrium exceeds that in the left. Defects high in the atrial septum (I., B) which do not involve the fossa ovalis are usually associated with anomalous right pulmonary veins connected to the junction of the superior vena cava and right atrium.[14] A defect in the region of the fossa ovalis may coexist. An anomalous connection of the pulmonary veins may coexist with any of the varieties of interatrial communication.

METHODS

Cardiac catheterization was carried out by the techniques described elsewhere. Seven of the patients, all less than 8 years of age, were anesthetized by means of tribromoethanol

(Avertin) given rectally, supplemented when necessary by intermittent intravenous injections of thiopental sodium. The doses of the 2 drugs were adjusted according to the size of the patient.[15] The unanesthetized adults received 1.5 gr. (0.1 Gm.) of seconal and 0.5 gr. (0.032 Gm.) of codeine orally before the procedure; the doses were reduced according to weight for the unanesthetized children.

Intravascular pressure transmitted through the catheter and systemic artery pressures were recorded by a photo-oscillographic assembly,[16] using specially adapted strain-gauge manometers.

When pressures between two locations in the heart and great vessels were to be compared, only those pressures recorded immediately before and just after withdrawal of the catheter from the one site into the other were evaluated.

Mean pulmonary and systemic arterial pressures were calculated by adding a third of the pulse pressure to the diastolic pressure. The average mean pressure obtained by planimetric integration of the pressure records in 10 patients did not differ significantly from the mean pressure obtained by this method.

An oximeter on each ear continuously recorded the oxygen saturation of peripheral arterial blood.[17] Samples of blood were drawn through a cuvette oximeter attached to a catheter for intermittent determination of oxygen saturation* at different locations in the heart chambers and great vessels. For recognition of left-to-right shunts, samples were drawn from the various chambers and vessels in rapid succession.

Intermittently during the procedure, samples of blood were drawn from the radial artery and chambers of the heart for determination of oxygen saturation by manometric technique.[18, 19]

Pulmonary (Q_P) and systemic (Q_S) blood flows† were calculated by the Fick principle from oxygen consumption (V_{O_2}) and oxygen content of samples of blood drawn from the radial (C_{saO_2}) and pulmonary arteries (C_{paO_2}), and venae cavae.[20] When the oxygen saturations of blood samples from radial and pulmonary arteries were nearly identical, the blood samples were compared precisely by passing them through the same oximeter. By this means differences of saturation of the order of 1 per cent could be measured with accuracy.

$$Q_P = \frac{V_{O_2}}{(C_{pvO_2} - C_{paO_2})} \quad ; \quad Q_S = \frac{V_{O_2}}{(C_{saO_2} - C_{mvO_2})}$$

Q_P = Pulmonary flow (liters per minute)

Q_S = Systemic flow (liters per minute)

V_{O_2} = Oxygen consumption (cubic centimeters per minute)

C_{pvO_2} = Oxygen content (cubic centimeters per liter) of pulmonary vein blood‡

C_{paO_2} = Oxygen content (cubic centimeters per liter) of pulmonary arterial blood

C_{saO_2} = Oxygen content (cubic centimeters per liter) of systemic arterial blood

C_{mvO_2} = Oxygen content (cubic centimeters per liter) of mixed venous blood

$$\frac{C_{svcO_2} + 2C_{ivcO_2}}{3} = C_{mvO_2}$$

Left ventricular stroke volume was calculated to equal $\frac{Q_s}{R}$.

R = cardiac rate at time that samples of blood were drawn for estimation of systemic flow.

Pulmonary and systemic flow and stroke volume were expressed also in relation to body surface of the individual (pulmonary flow index; systemic flow index; stroke volume index).

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*Values are expressed as a percentage of the oxygen capacity. These data could be expressed as volume of oxygen per 100 c.c. of blood by multiplying by the oxygen capacity.

†Symbols modified from "Standardization of Definitions and Symbols in Respiratory Physiology."[20]

‡Patient breathing room air, C_{pvO_2} = (98 per cent of oxygen capacity + 0.3) ×10. Patient breathing 100 per cent oxygen, C_{pvO_2} = (100 per cent of oxygen capacity + 2) ×10.

Shunts were calculated as follows, and expressed as per cent of either pulmonary or systemic flows:

Venoarterial (right-to-left) shunt expressed as per cent of systemic flow = $\frac{(Q_S - Q_P)}{Q_S} \times 100$

Arteriovenous (left-to-right) shunt expressed as per cent of pulmonary flow = $\frac{(Q_P - Q_S)}{Q_P} \times 100$

In the absence of an estimate of oxygen consumption, shunts were calculated as follows:

Venoarterial shunt expressed as per cent of systemic flow = $\frac{C_{pvO_2} - C_{saO_2}}{C_{pvO_2} - C_{mvO_2}} \times 100$

Arteriovenous shunt expressed as per cent of pulmonary flow = $\frac{C_{paO_2} - C_{mvO_2}}{C_{pvO_2} - C_{mvO_2}} \times 100$

Arterial dye-dilution curves were recorded by means of ear oximeters and simultaneously by a cuvette oximeter attached to a needle in the radial artery[21] following injection of Evans blue dye (T-1824) at various sites in the heart and great vessels.[22]

A venoarterial shunt was demonstrated by an abnormally short appearance time for the dye at the recording site and an abnormal initial deflection of the curve.[23] Prolongation of the disappearance slope of the curve confirmed the presence of an arteriovenous shunt.[24]

In those cases in which a right-to-left shunt was indicated by an abnormal indicator-dilution curve, but in which there was no decrease in oxygen saturation of peripheral arterial blood, the per cent of shunt was determined from the dye-dilution curve by a method described previously.[23]

The pulmonary resistance was calculated as follows:

$\frac{P_{Pm} \times 1{,}332 \times 60}{Q_P} = R_P$, where P_{Pm} is the mean pulmonary arterial pressure in millimeters of mercury measured at the time that the blood samples were drawn for calculation of pulmonary blood flow: 1,332 is the conversion factor from millimeters of mercury to dynes per square centimeter; Q_P is pulmonary blood flow in cubic centimeters per second; R_P is total pulmonary resistance expressed in dynes · second · cm.$^{-5}$.

CRITERIA FOR DIAGNOSIS

A diagnosis of atrial septal defect was made after cardiac catheterization when a left-to-right shunt had been demonstrated between the two atria or if a significant right-to-left shunt was present and a small left-to-right shunt was demonstrated inconstantly. The passage of the catheter from the right atrium to the left atrium without accompanying evidence of shunting was not considered necessarily diagnostic of an abnormal interatrial communication, since it is possible for a catheter to pass through a normal valve-competent foramen ovale.

The oxygen saturation of blood in the venae cavae and right atrium was determined by drawing samples in rapid succession through a cuvette oximeter. In an unanesthetized patient, the oxygen saturation of blood in the inferior vena cava is almost always higher than that in the superior vena cava so that whenever possible samples were obtained from both vessels. Variation in oxygen saturation occurs at different levels in the inferior vena cava, so that the tip of the catheter was positioned at the level of the diaphragm to insure as representative a sample as possible.

An arteriovenous shunt was considered present if the oxygen saturation of blood in the right atrium was demonstrated to be 2 or more per cent higher than the average oxygen saturation of the mixed venous blood.

In those cases in which arterial dye-dilution curves were recorded after injection of T-1824, the presence of a left-to-right shunt was verified by an abnormally long disappearance slope of the curve, indicating pulmonary recirculation of dyed blood.

After injection of dye into either cava, a short appearance time of the dye at the recording site with an abnormal initial deflection indicated a venoarterial shunt. Confirmation of the site at which the shunt was occurring was obtained by absence of an abnormal initial deflection when dye was injected into the right ventricle. The dye-dilution curve is the most accurate method of ascertaining the presence of a right-to-left shunt. However, in cases in which a dye-dilution curve was not obtained, the finding of a peripheral arterial oxygen saturation of less than 94 per cent with the patient breathing air, or the finding of less than 1 volume per cent of oxygen physically dissolved with the patient breathing 100 per cent oxygen, was considered evidence of a venoarterial shunt.

Conditions which cause arterialization of blood in the right atrium, and thus may be confused with atrial septal defect are: (1) common atrioventricular canal; (2) anomalous connection of a pulmonary vein or veins; (3) aneurysm of the posterior aortic sinus with rupture into the right atrium; (4) ventricular septal defect with insufficiency of the tricuspid valve; (5) ventricular septal defect opening into the right atrium; and (6) communication between coronary sinus and left atrium.

MATERIAL STUDIED

The hemodynamic data of those patients having had cardiac catheterization in our laboratory between December, 1947, and October, 1954, were reviewed. There were 121 cases in which a diagnosis was made of abnormal interatrial communication.

Patients who had associated pulmonary hypertension, anomalous connection of a pulmonary vein or mitral valve disease were included in the series because the atrial septal defect was the primary anomaly. Those whose condition was diagnosed as common atrioventricular canal were included because of the similarity of the hemodynamics of the two conditions. Those with other associated abnormalities were excluded because the interatrial communication was considered a part of a more complex anomaly (Table I).

TABLE I. INCIDENCE OF ANATOMIC DEFECTS IN 121 PATIENTS IN WHOM AN INTERATRIAL COMMUNICATION WAS A HEMODYNAMICALLY SIGNIFICANT LESION

	CASES	PER CENT OF TOTAL
Atrial septal defect, uncomplicated	44	36
Associated pulmonary hypertension	13	11
Associated anomalous pulmonary venous connection	6	5
Associated mitral valve disease	2	<1
Common atrioventricular canal	6	5
Total patients considered in this report	71	59
Other associated abnormalities		
Pulmonary stenosis	26	21
Ventricular septal defect	13	11
Tricuspid atresia	5	4
Ebstein's deformity of the tricuspid valve	3	2
Persistent truncus arteriosus	1	1
Patent ductus arteriosus	1	1
Coaretation of the aorta	1	1
Total	121	100

Twenty patients demonstrated a gradient of pressure across the pulmonary valve greater than 50 mm. of mercury and were excluded because this was considered indisputable evidence of stenosis of the pulmonary valve. An additional 6 patients had a pressure gradient greater than 20 mm. of mercury across the valve and were excluded as probably having organic stenosis of the pulmonary valve; a more detailed discussion of this matter will follow.

In 13 cases a ventricular septal defect was associated, and the catheterization data did not indicate that an atrioventricular type of lesion (persistence of a common atrioventricular canal) was present.

TABLE II. HEMODYNAMIC DATA IN 57 CASES OF ATRIAL SEPTAL DEFECT
(CASES ARE ARRANGED IN ORDER OF INCREASING PULMONARY BLOOD FLOW)

CASE	AGE (YR.)	PRESSURE (MM. HG)				BLOOD OXYGEN SATURATION (PER CENT)					ARTERIAL BLOOD FLOW (L./MIN./M.²)		SHUNT (PER CENT OF BLOOD FLOW)	
		R.A.	R.V.	P.A.	RADIAL ARTERY	I.V.C.	M.R.A.	S.V.C.	P.A. OR R.V.	RADIAL ARTERY	PULMONARY	SYSTEMIC	RIGHT-TO-LEFT	LEFT-TO-RIGHT
1	24	19/6	122/20	121/69	100/76	55	51	49	50	80	0.8	1.1	25	0
2	38	9/6	85/17	82/49		54	50	48	50	75	1.0	1.8	45	0
3	23	8/3	150/2	150/80	114/66	62	62	59	55	82	1.3	3.3	60	0
4*	54	6/3	84/3	84/34	127/71		55	56	55	83	1.5		35†	
5*	26	6/4	139/10	139/89	113/65	65	66	61	61	78	1.6	2.9	45	0
6	45	6/2	117/8	117/54	112/65	52	67	45	71	83	2.3	2.4	30	45
7	45	5/1	85/3		128/69	72	70	59	67	94	3.2	1.6	0	50
8	26	5/2	§	77/48	106/65	71	74	66	71	95	3.4	2.6	15	20
9‡	42	7/12	22/5	22/10	110/67	65	79	68	80	96	4.4	2.2	0	50
10	38	5/2	29/3	21/6	95/51	76	82	71	85	97	4.9	3.0	0	40
11	51	10/3	52/7	33/17	132/72	61	76	64	76	94	5.0	2.0	0	40
12	33	5/2	56/6	56/23	155/94	66	71	62	72	95	5.5	3.8	0	30
13	20	14/5	44/0	31/12	116/69	77	88	88	87	96	5.5	3.0	0	45
14	14	7/3	24/7	21/16	148/78	79	81	71	87	98	6.4		0	50
15‡	24	6/4	69/7	68/43	104/59	77	88	67	87	93	6.4	3.5	20	45
16	58	11/5	42/9	37/20	148/77	67	82	65	85	97	6.7	2.9	0	55
17	16	10/0	47/5	40/16	119/64		88	78	91	95	6.8	4.3	0	40
18	22	8/6	33/7	32/6	144/80	84	87	82	88	98	6.8	3.7	0	45
19	25	3/1	30/9	29/18	120/72	76	89	81	90	95	6.9	3.7	0	45
20	15	6/3	29/0	21/7	97/53	75	84	72	86	97	7.1	3.8	0	45
21‡	14	21/0	37/5	28/10	123/78	78	90	80	91	98	7.2	3.5	0	50
22	15	7/4	30/6		136/78	79	87	79	89	97	7.6	3.7	0	50
23	31	17/10	48/9	44/17	132/74	77	85	67	86	92	7.7	2.7	25	75
24	52	13/4	53/7	43/15	180/108	73	80	71	84	95	7.8	2.5	0	70
25	12	8/2	37/0	22/5	115/60	79	82	74	83	96	7.8	4.4	0	40
26	24	12/7	35/12	31/8	124/69	81	94	77	92	99	8.0	3.8	0	50
27*	47	23/16	114/9	114/39	101/74	79	87	59	87	97	8.1	2.5	0	70
28	47	9/1	37/3	34/24	113/66		95	80	93	98	8.3	2.8	0	65
29‡	33	9/2	31/2	26/9	94/57	80	87	64	88	97	8.4	3.0	0	60
30‡	46	4/–1	21/4	22/8	114/64		88	65	90	95	8.8	2.6	0	70
31	8	5/10	40/6	36/26	147/48	75	79	69	83	96	8.9	4.0	0	55
32	7	7/3	39/3	27/15	84/51		91	82	89	95	9.0	2.6	0	70
33	18	11/5	54/12			75	92	72	92	97	9.0	2.8	0	70
34	22	11/9	49/11	30/21	108/60	71	89	63	87	95	9.0	3.6	0	60

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35	19	7/3	30/4	21/8	120/72	83	86	76	85	97	9.2	4.7	0	50
36	24	10/7	36/9	36/13	134/72		92	69	91	96	9.4	2.5	0	75
37	8	7/1	49/12	31/21			89		90	98	9.7	4.4	0	55
38‡	5	5/2	24/4	20/7	92/57	81	91	75	92	96	9.8	4.2	0	55
39‡	26	6/1	37/2	30/15	135/75	83	89	73	91	96	10.3	4.4	15	55
40‡	19	11/5	31/6	21/9	116/58	84	85	76	93	98	10.6	3.1	0	70
41‡	23	8/3	36/3	32/10	125/79	84	91	70	90	99	10.9	3.9	0	65
42‡	40	7/4	31/0	28/8	100/45	83	89	75	90	98	11.5	3.3	0	70
43‡	13	5/1	28/6	25/10	90/60	80	87	76	91	97	13.1	5.2	0	60
44	38	12/3	36/2	32/6	112/69		89	65	90	96	13.3	2.3	0	80
45‡	30	7/3	27/2	25/9	101/54	88	94	81	94	97	13.3	4.2	5	70
46‡	27	15/7	29/7	29/14	116/65	88	95	68	93	98	13.8	3.0	10	80
47	37	10/5	41/9	28/15	108/62		84	74	88	97	14.4	3.3	0	75
48	13	11/6	44/9	29/16	107/65		89	76	92	95	14.4	2.6	0	80
49	20	11/6	36/11	33/15	115/68	81	87	77	92	95	14.7	4.4	0	70
50	26	7/4	43/5	27/16	107/62		93	76	92	97	14.9		0	75
51	36	6/2	130/5	121/57	130/73		89	81	93	97	16.7	5.6	0	60
52	4	6/2	54/7	41/26	83/54	78	90		90	86	16.9	5.0	0	70
53	45	7/2	34/2	33/9	127/78	86	94	65	93	95	17.4	3.7	0	80
54‡	27	12/6	40/6	35/17		88	94	66	93	99	20.7	5.6	0	75
55‖	3	6/2	28/5	22/10		76	88	73	89					60
56‖	27	26/15	78/8	78/14	112/72	37	44	35	39	75			40	10
57‖	26	10/8	108/13	108/61	109/68	72	74	65	74	86			45	20

*Diagnosis confirmed at necropsy.

†Right-to-left shunt by dye.

‡Surgical confirmation of diagnosis.

§Tachycardia while catheter was in right ventricle.

‖Data on oxygen consumption not obtained.

Key to abbreviations in Tables II and III: R.A. = Right atrium; R.V. = Right ventricle; P.A. = Main pulmonary artery; I.V.C. = Inferior vena cava; M.R.A. = Middle of right atrium; S.V.C. = Superior vena cava.

TABLE III. HEMODYNAMIC DATA IN 14 PATIENTS WITH ATRIAL SEPTAL DEFECT AND ASSOCIATED ANOMALY (CASES ARRANGED IN ORDER OF INCREASING PULMONARY BLOOD FLOW)

CASE	AGE (YR.)	PRESSURE (MM. HG)				BLOOD OXYGEN SATURATION (PER CENT)					ARTERIAL BLOOD FLOW (L./MIN./M.²)		SHUNT (PER CENT OF BLOOD FLOW)	
		R.A.	R.V.	P.A.	RADIAL ARTERY	I.V.C.	M.R.A.	S.V.C.	P.A. OR R.V.	RADIAL ARTERY	PULMONARY	SYSTEMIC	RIGHT-TO-LEFT	LEFT-TO-RIGHT
Anomalous Connection of Pulmonary Veins														
58	31	6/3	115/2	113/52	122/68	67	69	60	68	87	2.6	2.9	30	25
59*	32	11/5	36/13	29/13	124/62	84	91	93	93	98	6.8	3.4	0	50
60*	23	10/6	28/5	29/18	105/70	73	85	63	87	95	8.4	2.8	0	65
61*	30	25/20	55/21	55/30	116/80	81	89	68	87	96	8.9	4.8	0	45
62*	43	9/3	43/8	32/44	107/61	77	95	66	94	96	11.5	2.4	0	80
63*	22	7/0	41/8	28/12	129/72	85	96	79	95	98	14.2	3.1	0	75
Common Atrioventricular Canal														
64	9	7/3	101/3		95/71	71	71	65	87	89	3.1	3.1	30	25
65	5	7/3	32/7	33/17	91/62	71	73	68	87	96	6.4		10	60
66*	27	12/6	32/5	27/6	106/71	80	68	62	85	98	8.6	2.8	5	65
67	7†	13/8	40/7		85/59	83	86	81	88	98	10.9	4.1	10	60
68	35	7/4	26/4	25/9	115/57	78	82	62	91	94	11.6	3.5	10	70
69‡	27	7/3	142/1	137/66	106/69	63	71	64	72	81		3.7	50	25
Mitral Disease														
70*	37	18/13	107/5	107/18	138/99	54	62	52	61	96	3.0	1.6	0	50
71*	23	22/11	34/5	35/14	72/57	72	84	50	87	92	5.6	1.7	20	70

*Surgical confirmation of diagnosis.
†Dextrocardia.
‡Diagnosis confirmed at necropsy.

Since the diagnosis of mild organic stenosis of the pulmonary valve complicating atrial septal defect is based on somewhat arbitrary rules, and since the hemodynamic criteria for diagnosis of common atrioventricular canal have not been definitely established, it is possible that the selected group of cases of uncomplicated atrial septal defect includes some examples of the two conditions.

This study, then, is concerned with the 71 patients in whom the atrial septal defect was the primary congenital defect.

RESULTS

The vital statistics and pertinent hemodynamic data for these patients are given in Tables II and III.

Sex and Age.—Forty-nine patients were females, and 22 were males; a ratio of 2 to 1. The ages ranged from 3 to 58 years with more than half of the patients between 21 and 40 years of age (Fig. 1).

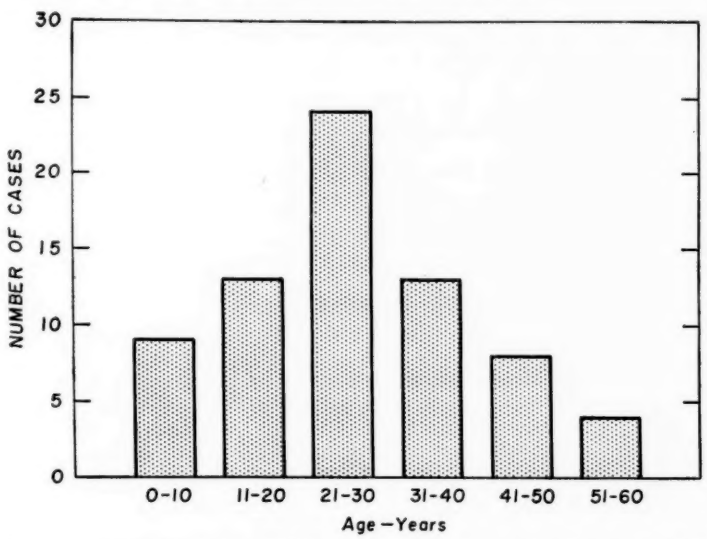

Fig. 1.—Frequency distribution according to age at the time of cardiac catheterization of the 71 patients with interatrial communication considered in this report.

Pulmonary Blood Flow.—The normal cardiac output for adults at rest, breathing air, as calculated by the Fick principle, has been found to average 3.4 liters per minute per square meter (range 2.8 to 5.3 L./min./M.^{2}).[25] Based on these values of expected normal pulmonary flow, the series was divided into groups of decreased, normal, moderately increased, and markedly increased pulmonary flow index (Table IV). The blood flow was determined while the patient was at rest and was breathing air, and the mean pulmonary blood flow for the group was 8.3 L./min./M.^{2}, two and a half times normal. In more than three quarters of the group the pulmonary blood flow was moderately to markedly increased. The seven patients with decreased pulmonary blood flow all had severe pulmonary hypertension.

Systemic Blood Flow.—The systemic blood flow at rest was within the range of normal in 69 per cent of the subjects in whom it was measured (Table IV). Only 5 of the group had more than a minimal decrease in systemic flow. All of these had pulmonary hypertension.

TABLE IV. DISTRIBUTION OF PULMONARY AND SYSTEMIC BLOOD FLOWS IN PATIENTS WITH INTERATRIAL COMMUNICATION

	BLOOD FLOW		
	L./MIN./M.^{2}	SYSTEMIC CASES	PULMONARY CASES
Decreased	Less than 2.0	5	7
	2.0-2.8	15	
Normal	2.8-5.3	42	7
Moderate increase	5.4-9.0	3	26
Marked increase	More than 9.0	0	27
Total		65	67

The stroke volume index has been calculated to range between 40 and 65 ml./beat/M.^{2} in normal adults studied in this laboratory while at rest and breathing air. The average for this group was 49 ml./beat/M.^{2} The stroke volume index of the left ventricle was calculated in 21 adults with atrial septal defect in our group who had normal systemic blood flow. The average stroke volume in this group was 44 ml./beat/M.^{2} Sixteen of these patients had stroke volumes between 40 and 60 ml./beat/M.^{2}, which is within the range of normal. The stroke volume of the remaining 5 was somewhat reduced and lay between 30 and 40 ml./beat/M.^{2}; 3 of these had uncomplicated atrial septal defects, 1 had severe pulmonary hypertension, and 1 had common atrioventricular canal. The average left ventricular stroke volume of 8 adult patients with systemic flow of less than 2.8 L./min./M.^{2} was calculated and was found to be 25.1 ml./beat/M.^{2}, with a range from 15.7 to 34.3 ml./beat/M.^{2} Two of the patients with low stroke volumes had uncomplicated atrial septal defect and had a stroke volume that was only minimally reduced, being greater than 30 ml./beat/M.^{2} The remaining 6 were either in cardiac failure or had severe pulmonary hypertension.

There was no correlation between the magnitude of the pulmonary and systemic blood flows in these patients. In patients with uncomplicated atrial septal defect, the systemic blood flow remained within the range of normal despite the magnitude of pulmonary flow.

Oxygen Saturation.—A comparison of oxygen saturation of samples of blood withdrawn in rapid succession from the inferior and superior venae cavae in unanesthetized patients revealed that in most the oxygen saturation was significantly higher in the inferior vena cava than in the superior vena cava (Fig. 2). The average difference was 7 per cent, a difference which is approximately that found in normal adult subjects.

The oxygen saturation of samples of blood drawn in rapid succession from the right ventricle and right atrium differed in many of the subjects (Fig. 3); however, the difference was not constant when sampling was repeated. In 2 patients, the oxygen saturation of samples of right ventricular blood was 6 to 8 per cent higher than that of right atrial blood, suggesting that perhaps a ventricular septal defect coexisted. These 2 patients were operated on for closure of the atrial septal defect, and the ventricular septum in each was found to be intact.

Four of the 6 patients with a diagnosis of common atrioventricular canal showed a consistent and significant increase in the oxygen saturation of blood in the right ventricle over that in the right atrium; the remaining 2 patients had only insignificant increases on repeated sampling.

Shunts.—From the data on blood oxygen saturation it was found that 49 patients (64 per cent) had shunts only in the left-to-right direction while at

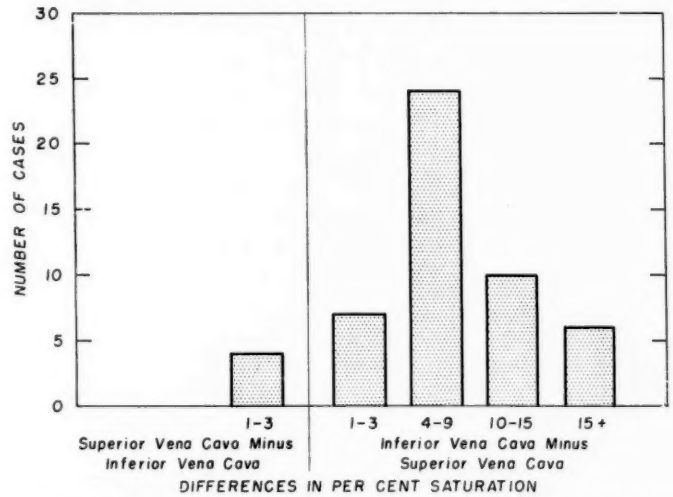

Fig. 2.—Frequency distribution of differences in oxygen saturation of inferior and superior caval blood in 51 of the patients studied while conscious. The determinations were made by means of a cuvette oximeter through which the samples of blood were drawn in quick succession (average interval between samples was 45 seconds). As in normal subjects the saturation of blood from the inferior vena cava exceeds that of superior vena cava by an average of 7 per cent. This relation does not hold under conditions of anesthesia. In a few cases the magnitude of this difference suggested that blood in the inferior vena cava was being contaminated by blood of high oxygen saturation, either due to an anomalous connection of a pulmonary vein to the inferior vena cava or due to reflux of shunted blood into the inferior cava itself.

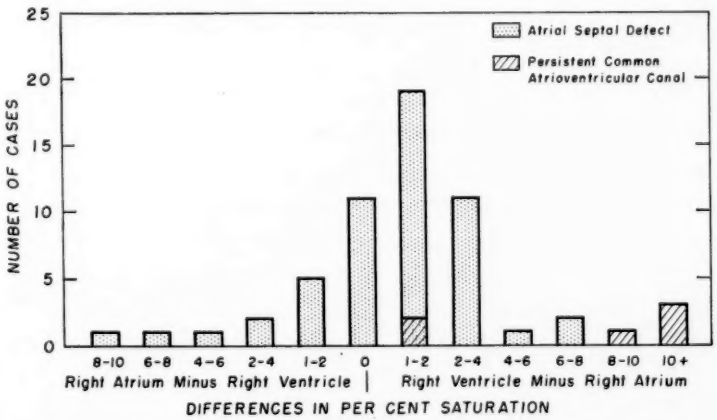

Fig. 3.—Frequency distribution of differences between the oxygen saturation of blood from the right ventricle and right atrium, in 58 of the patients. Samples were obtained successively from just within the right ventricle and from the right atrium close to the tricuspid valve (average interval between samples was ½ minute). Note the wide range of differences which may exist in patients without interventricular communication. However, the greatest differences were obtained in 4 of the patients with persistent common atrioventricular canal.

rest and breathing air, 17 (24 per cent) had bidirectional shunts through the defect, and 5 (7 per cent) had right-to-left shunts only. In the more recent catheterizations, small right-to-left shunts from the inferior and superior venae cavae have been demonstrated by the dye-dilution methods in the majority of cases so studied. Only in patients with pulmonary hypertension, however, has the right-to-left shunt been of sufficient magnitude to cause significant arterial desaturation.

More than 70 per cent of patients with right-to-left shunts whether of small or large magnitude showed preferential shunting of blood from the inferior vena cava. More than 70 per cent of those patients in whom dye curves were obtained after injection of dye into the right and left pulmonary arteries showed preferential left-to-right shunting of blood from the right lung. All the patients without pulmonary hypertension showed a left-to-right shunt, which averaged 55 (20 to 70) per cent of the pulmonary blood flow. The right-to-left shunt in these patients, as estimated from the dye-dilution curves, averaged 9 (0 to 20) per cent of the systemic blood flow.

Two of the patients (Cases 27 and 51, Table I) with severe pulmonary hypertension had pure arteriovenous shunts amounting to 70 and 60 per cent of the pulmonary blood flow, respectively.

The 6 patients with common atrioventricular canal all had left-to-right shunts at the atrial level; 2 had demonstrable shunts through both atrial and ventricular septal defects. Two of the 3 patients with dye curves for the right and left pulmonary arteries showed no significant preferential shunting of blood from the right lung. All 6 patients also had right-to-left shunts of 5 to 50 per cent of the systemic flow through the atrial defect, but no right-to-left shunt could be demonstrated through the ventricular defect.

The patient (Case 58) who had an anomalous right pulmonary vein which entered the superior vena cava and severe pulmonary hypertension had a predominant right-to-left shunt.

One patient with mitral stenosis (Case 71) had a large arteriovenous and a small venoarterial shunt through the atrial defect. The other patient with mitral disease (Case 70) had severe mitral insufficiency and an atrial defect of the valve-incompetent foramen ovale type.

Pulmonary Artery Pressure.—Pressure in the pulmonary artery was measured in 66 patients. In 45 it was normal or only minimally increased (systolic pressure 40 mm. of mercury or less). In 6 it was from 41 to 60 mm. of mercury, and the patients were classified as having moderate pulmonary hypertension. Ffteen patients had severe pulmonary hypertension with systolic pressures greater than 60 mm. of mercury (Fig. 4).

Of the 19 patients less than 21 years of age, only one, a child 4½ years of age, had moderate elevation of pressure in the pulmonary artery; the remainder had normal or only minimally elevated pressures. All the other patients with severe hypertension were adults, but only 3 were more than the age of 40 years (Tables II, III, and V).

There was no correlation between pressure and blood flow in the pulmonary arteries of patients who had a mean systolic pressure of 40 mm. of mercury or

less (Fig. 5). However, in the group with pulmonary hypertension (mean systolic pressure of more than 40 mm. of mercury in the pulmonary artery)

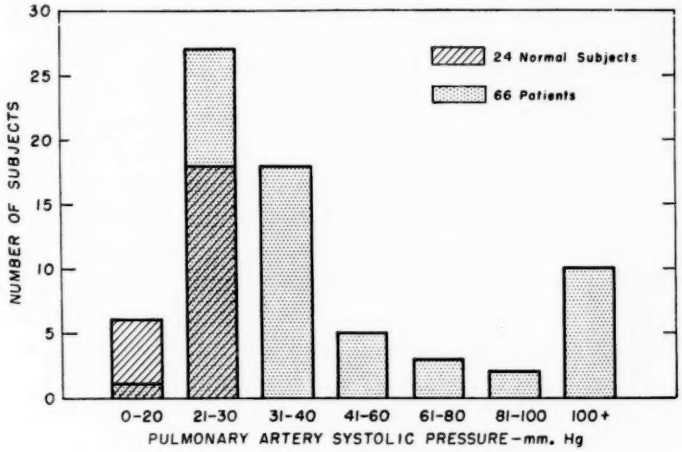

Fig. 4.—Frequency distribution of pulmonary artery systolic pressures in 66 patients with interatrial communication. The data obtained in 24 healthy subjects are included for comparison. Note that many of the patients have pressures similar to or slightly in excess of those obtained in the healthy subjects. Nevertheless 15 per cent of the patients had pulmonary artery systolic pressures in excess of 100 mm. of mercury.

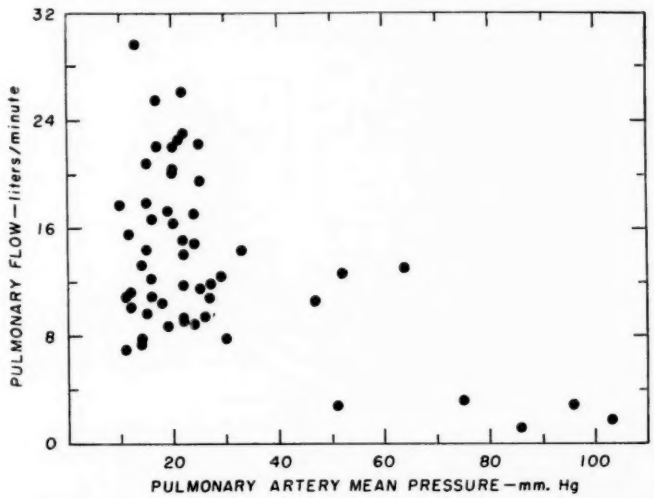

Fig. 5.—Relation of pulmonary blood flow to pulmonary artery mean pressure in 54 patients with interatrial communication. Note the apparent division of the data so that the flow values are almost independent of pressure in those patients with a mean pulmonary artery pressure of less than 30 mm. of mercury. In the patients with a mean pulmonary artery pressure in excess of 40 mm. of mercury there was an inverse relation between pressure and flow.

there was an inverse relation between pulmonary flow and pulmonary artery mean pressure. In Cases 15, 27, and 51 an increased pulmonary blood flow was maintained despite an elevated pulmonary artery mean pressure.

Pressure Gradient Across the Pulmonary Valve.—A significant pressure gradient occurred across the pulmonary valve in many of the patients, so that it was difficult to decide which ones had organic stenosis of the pulmonary

TABLE V. THE RELATION OF PULMONARY ARTERY PRESSURE TO AGE

SYSTOLIC PRESSURE IN PULMONARY ARTERY (MM. HG)	AGE OF PATIENTS IN YEARS			TOTAL	PER CENT
	<21	21-40	>40		
Less than 30	14	12	2	28	42
30 to 40	4	9	4	17	26
41 to 60	1	4	1	6	9
More than 60	0	12	3	15	23
Total	19	37	10	66	100

valve (Fig. 6). However, those patients with a systolic pressure gradient of more than 20 mm. of mercury had normal or low pulmonary blood flow, or a low systolic pressure in the pulmonary artery, and were excluded from the series as probably having organic stenosis of the pulmonary valve. The remaining patients with a significant gradient of pressure across the pulmonary valve had either high pulmonary artery pressure or a pulmonary flow which was

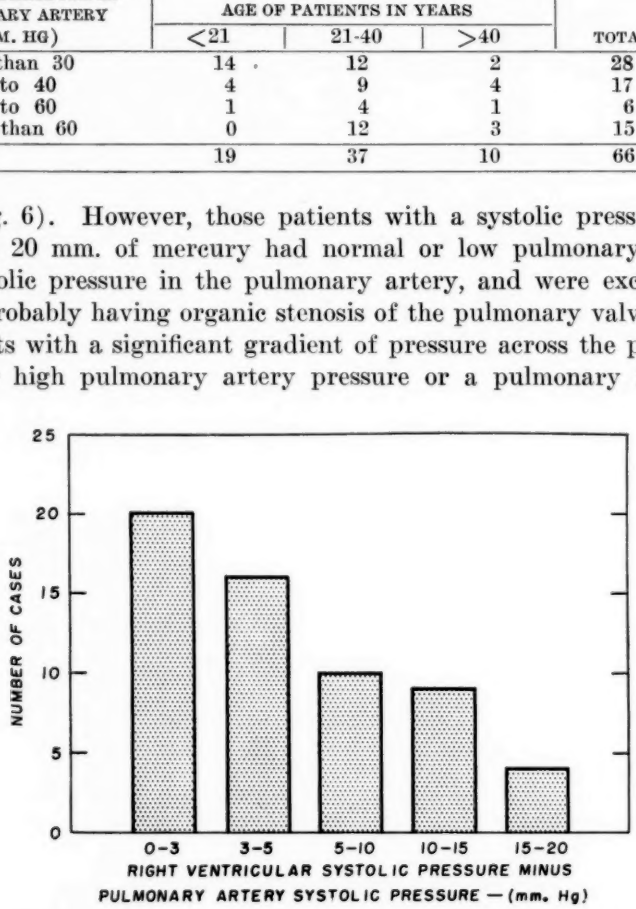

Fig. 6.—Frequency distribution of the magnitudes of the systolic pressure gradient across the pulmonary valve in 59 cases of interatrial communication. Measurements were only made from records of pressure obtained as the catheter tip was withdrawn across the pulmonary valve and in the absence of cardiac irregularities. In healthy subjects the average gradient was 2 mm. of mercury (range 0 to 7 mm. of mercury).[25]

greater than normal. Three of the patients with gradients of 10 mm. of mercury or more (Cases 40, 62, and 63) were operated on for closure of the atrial septal defect, and a normal pulmonary valve was demonstrated in each.

The magnitude of the gradient was unrelated to the height of the pulmonary artery pressure.

In individual patients there was no positive relationship between pulmonary blood flow and the systolic pressure gradient across the valve; however, there was a poor but statistically significant correlation between pulmonary flows and gradients for the entire group ($r = 0.4$).

Total Pulmonary Resistance.—In those patients without pulmonary hypertension, there was an inverse correlation between pulmonary blood flow and

pulmonary resistance. As the pulmonary flow increased, there was a corresponding decrease in calculated pulmonary resistance. In the majority of the subjects with severe pulmonary hypertension, pulmonary blood flow was decreased, and pulmonary vascular resistance was increased considerably.

Common Atrioventricular Canal.—The diagnosis of common atrioventricular canal was made in 6 cases (Cases 64 to 69, Table III). In all, there was a typical roentgenologic appearance of the catheter passing from the right atrium into the left ventricle. In 4 of the 6 there was a significant increase in the oxygen saturation of blood in the right ventricle as compared to that in the right atrium. In one patient (Case 69) who had severe pulmonary hypertension and no left-to-right shunt at ventricular level, the diagnosis of common atrioventricular canal was made at necropsy.

Cleft of the mitral or mitral and tricuspid valves accompanies a persistent common atrioventricular canal. Consequently, the pressure pulses of the right and left atria and the pulmonary artery wedge were examined for abnormally high V waves, which could have resulted from regurgitation of blood through one or both valves. Both of the cases in which the diagnosis was confirmed at operation had normal pressure pulses in the left and right atria.

Lutembacher's Syndrome.—Only one patient (Case 70) was given a diagnosis of mitral stenosis and this diagnosis was confirmed at operation. Of the 23 other patients in the series operated on for closure of the atrial septal defect, the mitral valve was normal in all but one (Case 71) in whom mitral insufficiency was present.

Anomalous Pulmonary Venous Connection.—Six patients had anomalous connection of one or more pulmonary veins. In all but one (Case 58) in whom operation was not performed, the diagnosis was confirmed at surgery.

COMMENT

This study of atrial septal defect and conditions causing nearly identical disturbances of the circulation confirmed certain general impressions. Females are more commonly affected than males. The relatively small proportion of children and adolescents studied, which is in contrast to the experience of this laboratory with many other forms of congenital heart disease, supports the impression that many patients enjoy good health during childhood.

The evidence is conclusive that in uncomplicated atrial septal defect the pulmonary blood flow is usually at least twice the systemic flow. Despite this substantial increase in pulmonary blood flow the pulmonary artery pressure remains normal or minimally elevated, and calculations of pulmonary vascular resistance give values within or below the range of normal. In adult patients with uncomplicated atrial septal defect, the systemic flow is normal. As the left-to-right shunt increases in magnitude with a consequent increase in pulmonary recirculation, a proportionate decrease in systemic flow might be expected. However, the data show no correlation between the magnitude of pulmonary and systemic flows, and indicate that many patients with uncomplicated atrial septal defect maintain a normal systemic flow and a normal left

ventricular stroke volume. Because of the normal difference of oxygen saturation (see below) between blood from the superior vena cava and inferior vena cava, use of the saturation of superior vena caval blood as representative of mixed venous blood for the calculation of systemic blood flow will result in a systematically lower value for systemic blood flow. This may be the explanation for the report of Dexter[26] that the left ventricular output is usually reduced in patients with atrial septal defects.

Certain observations will now be considered in somewhat greater detail.

Oxygen Saturation.—The higher oxygen saturation of blood in the inferior vena cava as compared to the superior vena cava is an important consideration when making the diagnosis of atrial septal defect during cardiac catheterization.

About half of all the blood returning to the right atrium is carried by the inferior vena cava; in normal subjects the oxygen saturation of this blood exceeds that returned by the superior vena cava by an average of 5.7 per cent (range −2 to +16 per cent).[27] Consequently, even in the presence of an intact atrial septum, a sample of blood drawn from the right atrium will usually have a slightly higher oxygen saturation than a sample drawn from the superior vena cava. The certain demonstration of an abnormally high oxygen saturation of right atrial blood is not usually a problem when it exceeds the saturation of superior caval blood by 10 per cent or more. When this is not the case, samples drawn in rapid succession from the inferior vena cava, the right atrium, and the superior vena cava, with the procedure repeated several times if necessary, will frequently enable a decision to be made as to the presence or absence of a left-to-right shunt into the atrium. Anomalous connection of the right pulmonary veins to the superior vena cava with associated interatrial communication has been seen in several patients. In this situation the oxygen saturation of superior caval blood will be equal to or exceed that of right atrial blood, and further samples must be taken upstream from the site of connection to determine the possible nature of such an anomaly. Clearly the demonstration of these differences is greatly facilitated by use of an instantaneous method of high relative accuracy such as the cuvette oximeter for the determination of oxygen saturation. The considerations outlined above are of extreme practical importance in obtaining the highest possible degree of accuracy in diagnosis in the group of patients who are candidates or potential candidates for surgical correction of their abnormalities.

The difference in oxygen saturation demonstrated between the blood in the right ventricle and right atrium is presumably the result of incomplete mixing of blood in the right atrium. The magnitude of such differences, which may frequently be considerable, is evident from Fig. 3. For example, if a sample of blood is drawn from near the coronary sinus and compared with a sample from the right ventricle, the oxygen saturation of blood in the right ventricle will appear greater and a ventricular septal defect might be suspected. Consequently, samples of right atrial blood should be drawn from a position as near to the center of the chamber as possible, and significant increases in oxygen

saturation of blood in the right ventricle should be demonstrable in several series of samples drawn in rapid succession from the two chambers before a diagnosis is made of an associated interventricular communication.

Shunts.—The direction of flow across an atrial septal defect is the result of differences in pressure in the right and left atria.[28] In most patients with uncomplicated atrial septal defects, the mean pressure is slightly higher in the left than in the right atrium, although the measurement of such differences is difficult. Such conditions were found in those of our patients in whom left and right atrial pressures were obtained nearly simultaneously.

A plausible explanation for the pressure difference between the atria is the lower filling pressure and greater distensibility of the right than of the left ventricle although Shaffer and associates[28] considered the pressure-volume characteristics of the atria and other factors more important. Although the mean pressure is higher in the left than in the right atrium, at certain times in the cardiac cycle, particularly early in atrial systole and immediately after atrial systole, there appears to be a reversal in gradient for a brief period. This reversal may explain the hemodynamically insignificant right-to-left atrial shunt which is frequently demonstrable by dye-dilution techniques.[5] The anatomy of the right atrium creates a natural channel for blood flow from the inferior vena cava to the foramen ovale. This may explain the finding that a greater proportion of inferior caval as opposed to superior caval blood shunts right to left in the brief period in which a right-to-left shunt occurs.

Gradient of Pressure Across the Pulmonary Valve.—Many patients with uncomplicated atrial septal defect have lower systolic blood pressure in the pulmonary artery than in the right ventricle. In those of our patients studied after repair of the defect, the pressure gradient was abolished as observed by Blount and co-workers.[29] In the group of 22 healthy subjects studied in this laboratory[25] the average systolic pressure gradient was 2.0 mm. with a range of 0 to 7 mm.

Many factors could be involved in producing the pressure gradient across the pulmonary valve. The relative diameter of the valve, as compared to the size of the right ventricular cavity and the degree of dilatation of the pulmonary artery, could affect the gradient. In the patient with atrial septal defect such a set of circumstances exists; a dilated right ventricle and pulmonary artery are separated by a normal pulmonary valve with resulting "functional pulmonary stenosis."

The height of pressure in the pulmonary artery was not related to the size of the gradient; however, there was a rough correlation between pulmonary blood flow and gradient. As the pulmonary flow increased, the gradient across the valve increased. It may be reasoned that the orifice of the pulmonary valve, although normal in diameter, represents a region of restriction to flow as the narrowest point in the pathway to the lungs. In this location high velocity and turbulence might well be anticipated. Since the systolic murmur of atrial septal defect is heard best over the region of the pulmonary valve, it seems logical to assume that it results from this functional stenosis of the pulmonary valve.

Pulmonary Hypertension.—The incidence of severe pulmonary hypertension is low in atrial septal defect,[9] and it is of interest that none of the patients less than 21 years of age had severe pulmonary hypertension. More recently a child, aged 1½ years, was found to have a pulmonary artery pressure of 54 mm. systolic and 36 mm. diastolic. At operation an atrial septal defect in the usual location was the only anomaly discovered and was repaired successfully. When pulmonary hypertension develops and symptoms become more severe, the patient is more likely to consult a physician and in turn have cardiac catheterization. Consequently, if pulmonary hypertension occurred as commonly in childhood as in later years, these patients would be sent for cardiac catheterization more frequently than those with uncomplicated atrial septal defect. For these reasons, it appears possible that pulmonary hypertension developed as a complication of atrial septal defect in some of the older patients, the duration of the left-to-right shunt possibly being one of the factors in its genesis. Exceptions to this generalization occur, for patients 60 years of age or more have been studied and found to have the clinical and hemodynamic features of atrial septal defect like those usually seen in young adults.

The pulmonary vessels of 2 of the patients in this series with pulmonary hypertension were examined. One (Case 27) had a high systolic pressure in the pulmonary artery and only moderately elevated diastolic pressure; the total pulmonary resistance was only moderately elevated; the pulmonary blood flow was high, and there was a pure left-to-right shunt. Microscopic examination of the lungs revealed medial hypertrophy of the muscular arteries with comparatively few vessels showing intimal changes.

In Case 5, systolic and diastolic pressures in the pulmonary artery were high; the total pulmonary resistance was markedly elevated; the pulmonary blood flow was reduced, and there was a right-to-left shunt through the atrial defect. Microscopic examination revealed extreme intimal thickening of the pulmonary arterioles with occlusion of vessels by connective tissue; there was also medial hypertrophy of the muscular arteries.

Recognition of Other Conditions Which May Simulate Atrial Septal Defect.—

Common atrioventricular canal: The differentiation of common atrioventricular canal at cardiac catheterization is important, since the surgical closure of this complex anomaly is best performed by open cardiotomy with extracorporeal circulation. Satisfactory repair of an atrial septal defect can be accomplished without the use of the latter technique.

Probably the most significant diagnostic finding may be the typical course taken by the catheter when passing from the right atrium into the left ventricle. When this finding is associated with a consistent, significant increase in oxygen saturation of blood in the right ventricle indicating an accompanying ventricular septal defect, there is great likelihood that a common atrioventricular canal is present.

Since the atrial defect in this type of lesion lies in a ventral position in the septum, there is usually a substantial posterior septal rim. Less preferential drainage of blood from the right lung might be expected than in defects lying

in the area of the foramen ovale. The number of cases studied are not sufficient to allow a conclusion to be drawn, but the indication is that absence of a preferential left-to-right shunting of blood from the right lung in the presence of a large pulmonary flow suggests that the interatrial communication may be low and ventral in the septum.

Anomalous pulmonary venous connection: Care must be taken at cardiac catheterization not to interpret the functional anomalous drainage of the right pulmonary veins frequently found in the usual case of atrial septal defect[3] as evidence for anatomic anomalous connection of the veins from one lung. Nor can the apparent passage of a catheter from the region of the right atrium into the right pulmonary veins be taken as certain proof of the presence of an anomalous connection.

However, anomalous venous connection of the right lung is associated very frequently with a defect in the atrial septum, and the diagnosis has been made in our cases during catheterization. The diagnostic aspects of these conditions have been considered in some detail in a previous publication.[4]

Certain cases of complete anomalous pulmonary venous drainage due to connection of all the pulmonary veins to the right atrium or to the superior vena cava with large pulmonary flows may closely simulate the clinical picture of atrial septal defect. Data on several such cases have recently been presented from this laboratory.[30]

Disease of the Mitral Valve.—The diagnosis of mitral stenosis is extremely difficult unless the tip of the catheter enters the left ventricle via the mitral valve. It is possible that in addition to the one patient in our series who had mitral stenosis, there might be others in whom the diagnosis was not made. The finding of normal pulmonary artery wedge and right atrial or left atrial (if entered) pressures in the presence of normal systemic flow is evidence that a hemodynamically significant degree of mitral stenosis does not exist.

If a clinically significant degree of mitral stenosis existed, the pressure level in the atria would be increased. If increased atrial pressure associated with reduced systemic blood flow is found in the absence of severe pulmonary hypertension, the possibility of mitral stenosis should be suspected. The diagnosis was made preoperatively on this basis in Case 71 reported in this series.

In two patients one (Case 27) with and one (Case 21) without pulmonary hypertension, who had very high pressures in the right atria, the mitral and tricuspid valves were found to be normal. In Case 21 the systemic flow was 3.5, and in Case 27 it was 2.5 L./min./M.^{2}

It is significant, however, that of the 24 patients of this series operated on, only one (Case 71) had mitral stenosis and no other patient has been found in 45 additional patients studied and operated on since the series was closed. The conclusion is that mitral stenosis is an infrequent complication of atrial septal defect.

SUMMARY

An interatrial communication was found as a significant lesion in a total of 121 patients studied up to 1954 in the cardiac catheterization laboratory of

the Mayo Clinic. Of these patients, 44 were considered to be examples of uncomplicated atrial septal defect, 13 had associated pulmonary hypertension, 6 had anomalous connection of one or more pulmonary veins, 6 had persistent common atrioventricular canals, and 2 had mitral valve disease. The hemodynamic data obtained from these 71 patients form the basis for this report. In the patients without pulmonary hypertension the pulmonary blood flow was greatly increased (average 8.3 L./min./M.^{2}) due to the presence of left-to-right shunting of considerable degree. In a number of these patients it was demonstrated that of the blood shunted left to right, a greater proportion originated from the right lung than from the left lung. Small right-to-left shunts without hemodynamic significance were shown to exist frequently, and of the blood shunted right to left a greater proportion was of inferior caval than of superior caval origin. In these patients the systemic blood flow was normal. The majority of patients with severe pulmonary hypertension had pulmonary and systemic flows of reduced magnitude. In 2 patients with severe pulmonary hypertension a large pulmonary blood flow was calculated.

Patients with anomalous connection of one or more pulmonary veins or with common atrioventricular canal may show the same fundamental circulatory disorder as patients with an atrial septal defect of the usual type. The frequency and practical importance of each of these lesions are compelling reasons that they be sought and diagnosed preoperatively. Highly suggestive evidence for each of these lesions may be obtained from critical analysis of the oxygen saturation of separate series of samples of blood drawn from different locations in the right ventricle, right atrium and both venae cavae. Differences of some magnitude may exist between samples drawn at random from these sites, but considerable significance may be placed on a difference that is consistently demonstrated.

A significant correlation was demonstrated between the pressure gradient across the pulmonary valve frequently found in these patients and the magnitude of the pulmonary blood flow. The gradient did not appear to be associated with a pathologically significant degree of pulmonary stenosis.

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THE EFFECT OF HEXAMETHONIUM UPON THE PULMONARY VASCULAR RESISTANCE IN MITRAL STENOSIS

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NUMEROUS investigations have been carried out in an effort to establish and delineate a neurogenic control of the pulmonary circulation in man.[1, 2] It has been postulated that there are pulmonary "arterioles" which have a sphincteric action and are under the control of the autonomic nervous system. Their constriction in various pathologic states has been stated to be a factor in the causation of pulmonary hypertension, and the extent that they participate in the regulation of pulmonary arteriolar resistance[3, 4, 14] has been studied by the administration of various autonomic nervous system blocking agents. One such agent is hexamethonium chloride, which is a ganglionic blocking agent and in adequate doses presumably completely inhibits the action of both the sympathetic and parasympathetic nervous systems.[5, 6]

The effect of hexamethonium in lowering the systemic blood pressure has been extensively studied.[7] In addition, its action in patients with pulmonary hypertension has recently come under investigation. Its hypotensive action has been studied in patients with pulmonary emphysema, and in those with heart failure.[8-10] Some authors have come to the conclusion that hexamethonium and similar ganglionic blocking agents have a relaxing effect upon the pulmonary arterioles,[2, 10] while others believe that the lowering of the pulmonary artery and left atrial pressures can be explained by other mechanisms, such as the pooling of blood in the systemic venous circuit and that no direct action of this drug upon the pulmonary vasculature need be postulated.[4, 11-13]

The following report deals with the hemodynamic effects of hexamethonium in patients with pulmonary hypertension, the majority of whom had mitral stenosis. The effects of this drug upon the pulmonary circulation have been studied both in the resting state and during exercise in order to determine in greater detail its effect upon the pulmonary vascular resistance.

METHODS AND MATERIALS

Fifteen patients with pulmonary hypertension were studied by the usual cardiac catheterization technique and in the supine position. Thirteen had mitral stenosis, one had pulmonary hypertension of unknown etiology, and one had lupus erythematosus with

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This study was supported in part by the United States Public Health Research Grant H-1208(C2).
Received for publication Dec. 27, 1956.
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pulmonary hypertension. The cardiac output, brachial artery, pulmonary artery, and pulmonary artery wedge pressures were determined at rest in all and during exercise in 14 of the 15 patients. The patients were in a fasting state and were allowed to rest upon the fluoroscopy table 15 minutes before the start of the procedure. The brachial artery needle was then put in place and the pulmonary artery catheterized, and the pressures recorded. An additional 10 minutes elapsed before the collection of the expired air and pulmonary and brachial artery blood samples for the determination of the resting cardiac output. Pulmonary and brachial artery pressures were recorded at the middle and near the end of this 3-minute period. Immediately after termination of the expired air collection, the pulmonary artery pressure was recorded and the pulmonary artery wedge pressure (PAWP) was measured. The catheter tip was advanced as far out into the peripheral pulmonary tree as possible, and wedged there. While recording the PAWP the catheter tip was withdrawn into the pulmonary artery. This maneuver was repeated several times. Approximately 5 minutes later exercise was begun after taking control pressure recordings. The "warm-up" period averaged 2½ minutes and ranged from 2 to 3 minutes, during which time the patients pedaled at constant speed a bicycle attached to the foot of the fluoroscopy table. The length of this period and the rate of exercise varied with each patient according to his exercise tolerance. A 3-minute expired air collection was then taken for the determination of oxygen consumption. Pulmonary and brachial artery blood samples were drawn during the second minute. Brachial and pulmonary artery pressures were recorded at 1-minute intervals during exercise. A pulmonary artery pressure was recorded and then immediately afterward the PAWP was measured, just after the end of the expired air collection while the patient was still exercising.

The patients were then allowed to rest, and pressure recordings continued at intervals until the pulmonary artery pressure had returned to the pre-exercise resting level, which usually occurred in 10 to 15 minutes. Hexamethonium administration was started 5 to 10 minutes later, after control pressures had been recorded. This drug was administered via the cardiac catheter at the rate of 1 mg. per minute for the first 10 minutes; the rate was doubled if no fall in systemic pressure had occurred, and if the patient was relatively insensitive it was next given in doses of 5 mg. per minute. Pressures were measured at 1-minute intervals. When the pulmonary artery pressure remained for 4 to 5 minutes at the lowest level that could be attained a resting cardiac output was determined and pressure measurements recorded as above. Hexamethonium administration was continued during this period. Five of these patients were then immediately exercised, during which time pressure measurements were made, and the cardiac output determined as above. The same rate and length of time of exercise were employed in each instance as before hexamethonium had been given. The amount of work done was estimated by the amount of oxygen consumed.

The average dose of hexamethonium chloride given was 45 mg., and ranged between 15 and 75 mg. Side effects were few, and generally consisted of slight blurring of vision, and nausea in a few instances. Dizziness and a sharp drop in blood pressure occurred in all patients upon sitting up.

The blood oxygen contents were measured by the Van Slyke and Neill technique. The oxygen and carbon dioxide of the expired air were determined by the Roughton-Scholander method. Pressures were recorded with Statham strain gauges and a Hathaway oscillograph.

DEFINITIONS

1. The systemic or total peripheral resistance is the resistance offered to blood flow from the left ventricle:

$$\text{TPR} = \frac{\text{Mean Brachial Artery Pressure in mm. Hg} - \text{O}}{\text{Cardiac Output in c.c./sec.}} \times 1{,}332$$

2. The total pulmonary resistance is defined as being the resistance offered to blood flow from the right ventricle by the structures in the vascular pathway between the

TABLE I. HEMODYNAMIC DATA OF FIFTEEN PATIENTS WITH PULMONARY

Patient Age B.S.*	Condition	Amount Hex. Admin. (mg.)	Brachial Art. Press. (mm. Hg) S	D	M	Pul. Art. Press. (mm. Hg) S	D	M	Pul. Cap. Press. (mm. Hg)	Cardiac Index (L./min./M.²)	Total Pul. Resist.	Pul. Arteriolar Resist.
1. B. J.	Rest		112	62	79	114	58	77	—	2.80	1,545	—
19 1.43	Hex.	15	58	36	43	82	61	68	—	2.67	1,425	—
2. J. G.	Rest		127	73	91	50	26	34	25	2.60	550	146
26 1.93	Hex.	50	87	55	66	40	22	28	19	2.20	541	174
3. J. T.	Rest		120	55	77	85	52	63	24	3.40	984	610
23 1.49	Hex.	100	100	45	67	66	39	48	24	3.10	835	418
4. J. L.	Rest		140	81	101	85	46	59	31	2.53	1,008	479
45 1.84	Hex.	55	110	67	81	63	36	49	27	2.68	794	356
5. L. S.	Rest		125	70	88	82	44	57	32	3.00	887	342
25 1.76	Hex.	50	113	68	83	62	40	47	—	3.30	652	—
6. D. C.	Rest		240	117	158	108	60	76	43	2.49	1,468	638
29 1.66	Hex.	20	119	74	89	72	42	52	25	2.51	995	517
7. M. C.	Rest		134	65	88	71	34	46	—	3.25	546	—
29 2.07	Hex.	55	92	56	68	44	29	34	—	2.25	584	—
8. I. B.	Rest		132	82	98	55	25	35	6	2.49	653	541
44 1.72	Hex.	25	110	71	84	25	13	17	5	2.58	306	217
9. M. D.	Rest		133	73	93	51	24	33	22	2.03	788	265
28 1.65	Hex.	40	102	61	75	39	25	30	25	2.70	541	90
10. H. L.	Rest		185	102	130	126	63	84	16	4.36	944	180
56 1.63	Hex.	50	120	76	91	80	38	52	—	3.64	700	—
11. K. P.	Rest		117	75	89	94	62	73	23	1.89	1,700	1,167
42 1.82	Hex.	25	103	76	85	80	54	63	21	1.73	1,600	1,067
12. W. B.	Rest		118	64	81	106	49	68	31	2.90	968	527
33	Hex.	30	98	58	71	54	32	39	15	2.80	581	358
13. G. R.	Rest		133	78	96	40	18	29	18	1.79	611	231
48 2.12	Hex.	50	103	73	83	31	18	22	20	2.68	310	17
14. B. C.	Rest		134	77	96	45	22	30	25	2.99	424	70
34 1.89	Hex.	27	100	60	73	28	17	22	20	3.23	287	26
15. J. S.	Rest		108	62	77	42	17	25	22	2.53	471	56
28 1.89	Hex.	75	100	62	75	38	21	27	16	2.32	551	220

*B.S. = Body surface area in M.²
†Kg. m./min./M.²
For key to abbreviations see footnote to Table II.

pulmonary artery and the left ventricle, and includes the resistance offered by pulmonary vasculature and by the mitral valve. Total pulmonary resistance =

$$\frac{\text{Mean Pulmonary Artery Pressure in mm. Hg} - \text{O}}{\text{Cardiac Output in c.c./sec.}} \times 1{,}332$$

3. The pulmonary arteriolar resistance is an estimation of the resistance to blood flow offered by the entire pulmonary vasculature from the pulmonary artery to the left atrium. The so-called "pulmonary capillary" or pulmonary artery wedge pressure (PAWP) is in reality a close approximation to the left atrial pressure, and not the pressure in the capillaries of the lungs. The pressure gradient under consideration, therefore, exists between the pulmonary artery and the left atrium. Pulmonary arteriolar resistance =

$$\frac{\text{Mean Pulmonary Artery Pressure in mm. Hg} - \text{PAWP in mm. Hg}}{\text{Cardiac Output in c.c./sec.}} \times 1{,}332$$

HYPERTENSION AT REST BEFORE AND AFTER HEXAMETHONIUM ADMINISTRATION

Total Periph. Resist.	Pulse Beats (per min.)	Stroke Index (c.c./min. /M.²)	Work R.V. (Kg. m.†)	Work L.V. (Kg. m.†)	Br. Art. O_2 (Sat. %)	Pul. Art. O_2 (Sat., %)	O_2 Consump. (c.c./ min.)	Diagnosis
1,584	110	25.5	2.9	3.0	93.1	66.1	189	Mitral stenosis
900	115	23.2	2.5	1.6	82.7	55.3	184	
1,472	93	28.0	1.2	3.2	94.1	67.7	272	Mitral stenosis
1,275	111	19.8	0.8	2.0	88.7	60.0	247	
1,200	90	37.8	2.9	3.6	93.2	69.5	218	Mitral stenosis
1,165	90	34.5	2.0	2.1	95.9	68.5	226	
1,728	95	26.7	2.0	3.5	90.2	57.6	282	Mitral stenosis
1,312	125	21.4	1.8	3.0	87.6	57.7	273	
1,370	82	36.6	2.3	3.9	96.0	69.0	246	Mitral stenosis
1,150	120	27.5	2.1	2.7	90.2	61.2	296	
3,052	96	26.0	2.6	5.4	81.7	49.5	247	Mitral stenosis
1,895	73	34.7	1.8	3.0	79.0	52.8	203	
1,044	100	32.5	2.1	3.9	96.2	70.9	303	Mitral stenosis
1,167	120	18.8	1.0	2.1	91.2	60.5	276	
1,380	83	30.0	1.2	3.3	90.9	62.0	229	Lupus
1,154	110	23.5	0.6	2.9	95.1	64.7	246	erythematosus
2,218	85	23.9	0.9	2.6	96.8	64.5	212	Mitral stenosis
1,350	88	30.7	1.1	2.8	93.5	68.6	217	
1,460	100	43.6	5.0	7.7	81.4	67.6	255	Pul. hypertension. Unknown etiology
1,225	97	37.6	2.6	4.5	86.5	70.0	255	
2,076	84	22.5	1.9	2.3	78.5	51.8	219	Mitral stenosis
2,158	112	15.5	1.5	2.0	84.5	53.2	236	
1,152	116	25.0	2.7	3.2	89.2	60.7	269	Mitral stenosis
1,057	110	25.5	1.5	2.7	84.0	54.3	262	
2,020	55	32.6	0.7	2.3	94.7	67.1	199	Mitral stenosis
1,168	88	30.5	0.8	3.0	95.3	70.7	266	
1,357	77	38.8	1.2	3.9	97.4	72.4	268	Mitral stenosis
957	110	29.4	1.0	3.2	93.2	71.1	255	
1,448	58	43.7	0.9	2.6	91.7	61.3	246	Mitral stenosis
1,530	82	28.3	0.8	2.4	92.4	60.1	241	

All resistances are expressed in absolute units (A.U.) the dimensions of which are dynes sec. cm.$^{-5}$.

The significance of the changes in pressure, resistance, and blood flow was evaluated statistically by Student's "t" test, as applied to the absolute values (not the percentage changes).

RESULTS

Resting State.—The following hemodynamic changes occurred in 15 patients.

Pressures: A decrease in the brachial artery pressure occurred in all patients (Tables I and II), and was statistically very significant.

TABLE II. MEAN VALUES IN FIFTEEN PATIENTS WITH PULMONARY HYPERTENSION AT REST BEFORE AND AFTER HEXAMETHONIUM ADMINISTRATION

	B.A.P. (mm. Hg) S/D	B.A.P. (mm. Hg) M	P.A.P. (mm. Hg) S/D	P.A.P. (mm. Hg) M	Pul. Cap. Press. (mm. Hg)	C.I. (L./min./M.²)	Total Pul. Resist.	Pul. Arteriolar Resist.*	Total Periph. Resist.	Ht. R. (beats/min.)	S.I. (c.c./min./M.²)	Wk. R.V. (Kg. m./min./M.²)	Wk. L.V. (Kg. m./min./M.²)	Br. Art. O_2 Sat. (%)	P.A. O_2 Sat. (%)	A-V O_2 Diff. (Vol. %)	O_2 Consump. (c.c./min.)
Before	137/76	96	80/40	53	24*	2.70	884	430	1,638	88	31.3	2.3	3.7	91.0	63.8	5.22	244
After hex. (45 mg.) av. dose	101/62	69	54/32	40	20	2.69	713	314	1,298	103	26.7	1.4	2.7	89.3	61.9	5.28	228
Mean per cent change	-26/-18	-28	-32/-20	-25	-17	0	-19	-27	-21	+15	-15	-39	-27	-1.9	-3.0	-1	-6
Statist. significance	$t = 4.63$ $p = 0.01$		$t = 5.22$ $p = 0.01$		$t = 2.05$ $p =$ sl. < 0.05		$t = 2.33$ $p = 0.05$	$t = 2.08$ $p =$ sl. < 0.05	$t = 3.41$ $p = 0.01$	$t = 3.06$ $p = 0.01$							

*Measured in 11 patients.

Key to abbreviations: B.A.P. = brachial artery pressure; P.A.P. = pulmonary artery pressure; Pul. Cap. Press. = pulmonary capillary pressure measured in 11 patients; C.I. = cardiac index; Total Pul. Resist. = total pulmonary resistance; Pul. Arteriolar Resist. = pulmonary arteriolar resistance; Total Periph. Resist. = total peripheral resistance; Ht. R. = heart rate; S.I. = stroke index; Wk. R.V. = work of right ventricle; Wk. L.V. = work of left ventricle; Br. Art. O_2 Sat. = brachial artery oxygen saturation; P.A. O_2 Sat. = pulmonary artery oxygen saturation; A-V O_2 Diff. = A-V oxygen difference; O_2 Consump. = oxygen consumption.

The pulmonary artery pressure decreased in all instances, and the average mean pulmonary artery pressure fell by approximately the same proportion as the average mean brachial artery pressure. This decrease was very significant. The mean pulmonary artery pressure decreased to one-half of the control level in Patient 8 (I. B.) with but a slight decrease in the systemic blood pressure, but this was the only instance of such a marked differential decrease. A few patients were quite sensitive to the hypotensive effect of hexamethonium (Patients 1, 6, and 8). However, about 50 mg. was necessary to demonstrate a noticeable effect in the majority. Others (Patients 3 and 15) showed but a slight reaction to the administration of even 75 mg. or more of hexamethonium.

The average "pulmonary capillary" (left atrial) pressure decreased by 17 per cent in the 11 patients where measurement was possible. However, this decrease was barely significant ($t = 2.05$, p slightly less than 0.05). In some instances there was no change (Patients 3 and 8), or a slight increase (Patients 9 and 13), but in the majority a decrease in the "pulmonary capillary" pressure (PAWP) occurred.

Cardiac index: There was no change in the average cardiac output after the administration of hexamethonium as compared to the control period. However, in 2 patients (9, 13) there was an increase in cardiac output, while the output decreased in Patients 7 and 10.

Resistance: The average systemic resistance (TPR) decreased by 21 per cent following the administration of hexamethonium. The total pulmonary resistance decreased by approximately the same percentage. However, the pulmonary arteriolar resistance decreased by 27 per cent. This decrease was barely significant statistically ($t = 2.08$, p slightly less than 0.05).[15] There was no correlation between the extent of decrease in the pulmonary arteriolar resistance and the drop in total pulmonary resistance in the relatively small number of cases in this series.

Pulse: The average heart rate increased by 15 per cent over the control level, which was significant statistically. Because of the increase in heart rate the average stroke index decreased after hexamethonium administration.

Work load: The average work load of the right ventricle decreased to a greater degree than that of the left ventricle. An increase in cardiac output and a decrease in pulmonary artery pressure occurred in Patients 9 and 13, while in Patient 15 they both remained constant as compared with the control levels. The work of the left ventricle decreased in all patients.

Blood gas contents and oxygen consumption: There was no significant change in either the average pulmonary arterial blood oxygen content or the A-V oxygen difference. The average oxygen consumption did not change following the administration of hexamethonium.

Summary: The administration of hexamethonium to these patients at rest and in the supine position resulted in a decrease in the mean brachial artery pressure and an approximately equal percentage decrease in the mean pulmonary artery pressure (Tables I and II). The decrease in left atrial pressure was not significant. The cardiac output remained constant. The decrease in

TABLE III. HEMODYNAMIC DATA AT REST AND DURING EXERCISE, BOTH

Patient Age B.S.*	Dose Hex.	State	Brachial Art. Press. (mm. Hg) S	D	M	Pul. Art. Press. (mm. Hg) S	D	M	Pul. Cap. Press. (mm. Hg)	Cardiac Index (L./min./ M.²)	Total Pul. Resist. (A.U.†)
K. P. 42 1.82	Control	Rest	117	75	89	94	62	73	23	1.89	1,700
		Ex.	138	87	104	134	66	95	30	2.38	1,750
	Hex. 25 mg.	Rest	103	76	85	80	54	63	21	1.73	1,600
		Ex.	109	77	88	109	74	86	27	2.25	1,678
W. B. 33 1.90	Control	Rest	118	64	81	106	49	68	31	2.90	968
		Ex.	185	85	118	106	74	103	35	3.90	1,106
	Hex. 30 mg.	Rest	98	58	71	54	32	39	15	2.80	581
		Ex.	122	73	89	127	63	84	37	3.30	1,067
G. R. 48 2.12	Control	Rest	133	78	96	40	18	29	18	1.79	611
		Ex.	150	92	111	86	45	59	34	3.27	679
	Hex. 27 mg. 67 mg.‡	Rest	103	73	83	31	18	22	20	2.68	310
		Ex.	123	80	94	63	41	48	30	2.20	824
B. C. 34 1.89	Control	Rest	134	77	96	45	22	30	25	2.99	424
		Ex.	170	95	120	90	50	66	40	4.37	639
	Hex. 75 mg.	Rest	100	60	73	28	17	22	20	3.23	287
		Ex.	100	60	73	58	33	41	34	3.53	492
J. S. 28 1.69	Control	Rest	108	62	77	42	17	25	22	2.53	471
		Ex.	112	60	79	82	38	53	30	3.55	712
	Hex. 75 mg.	Rest	100	62	75	38	21	27	16	2.32	551
		Ex.	103	60	74	55	32	40	27	3.28	577

*B.S. = Body surface area in M.²
†A.U. = Absolute units.
‡40 mg. additional hexamethonium given before cardiac output determined during exercise.
For key to abbreviations see footnote to Table II.

the total peripheral resistance and total pulmonary resistance were of the same magnitude. The percentage decrease in the pulmonary arteriolar resistance was somewhat greater. The pulse rate increased by 15 per cent. The work of the right and left ventricles was appreciably reduced.

Exercise.—Five of the 15 patients were also studied during exercise both before and after the administration of hexamethonium (Tables III and IV).

Exercise prior to hexamethonium administration resulted in an increase in the average mean brachial artery, pulmonary artery, and pulmonary capillary pressures. The cardiac output increased 42 per cent. The total peripheral resistance remained constant, but the total pulmonary and pulmonary arteriolar resistances increased significantly in all 5 instances, the latter by a greater percentage than the former. The increase in the work of the right ventricle was almost double that of the left ventricle.

The effect of exercise upon the circulation was altered following the administration of hexamethonium. There was insignificant increase in the average mean brachial artery pressure during exercise as compared to the resting state. The average mean pulmonary artery pressure again increased markedly with exercise. The percentage increase in the average pulmonary capillary pressure over the resting level was greater following hexamethonium than before it was given. The average cardiac output increased by only 14 per cent,

BEFORE AND AFTER HEXAMETHONIUM CHLORIDE ADMINISTRATION

Pul. Arteriolar Resist. (A.U.)	Total Periph. Resist. (A.U.)	Pulse (beats/ min.)	Stroke Index (c.c./min. /M.²)	Work R.V. (Kg.m. /min./M.²)	Work L.V. (Kg.m. /min./M.²)	Brachial Art. O_2 Sat.	Pulmonary Art. O_2 Sat.	O_2 Consump. (c.c./min.)
1,167	2,076	84	22.5	1.9	2.3	78.5	51.8	219
1,200	1,917	112	21.2	3.1	3.4	79.1	41.1	393
1,067	2,158	112	15.5	1.5	2.0	84.5	53.2	236
1,152	1,717	124	18.2	2.6	2.7	83.9	41.1	419
527	1,152	116	25.0	2.7	3.2	89.2	60.7	269
730	1,267	140	27.8	5.5	6.3	91.7	35.0	709
358	1,057	110	25.5	1.5	2.7	84.0	54.3	262
596	1,130	142	23.3	3.8	4.0	86.7	34.6	550
231	2,020	55	32.6	0.7	2.3	94.7	67.1	199
288	1,298	93	35.2	2.6	4.9	91.6	42.5	648
28	1,168	88	30.5	0.8	3.0	95.3	70.7	266
309	1,613	118	18.6	1.4	2.8	97.0	48.6	625
70	1,357	77	38.8	1.2	3.9	97.4	72.4	268
252	1,163	116	37.7	3.9	7.1	96.0	37.7	916
26	957	110	29.4	1.0	3.2	93.2	71.1	255
84	875	117	30.2	2.0	3.5	94.9	40.6	690
56	1,448	58	43.7	0.9	2.6	91.7	61.3	246
309	1,060	75	47.4	2.6	3.8	90.4	41.1	560
220	1,530	82	28.3	0.8	2.4	92.4	60.1	241
188	1,068	94	34.9	1.8	3.4	88.1	44.9	402

which was significantly less than before hexamethonium was given. The average total peripheral resistance again remained constant. There was still an appreciable and now equal increase in the average total pulmonary and pulmonary arteriolar resistances during exercise following hexamethonium administration. The percentage increase in the average total pulmonary resistance was greater than before hexamethonium. This change was barely significant statistically. The average percentage increase in the pulmonary arteriolar resistance was now about the same as the average percentage increase in the total pulmonary resistance. The pulmonary arteriolar resistance, however, increased in only 3 of the 5 instances, and this increase was not statistically significant. The increase in the right ventricular work caused by exercise was appreciable and was several times greater than the increase in the left ventricular work.

The hemodynamic data obtained during exercise before hexamethonium was given can be compared to the findings after hexamethonium administration (Table IV). The average absolute mean pressure levels reached in the brachial artery and pulmonary artery were less after giving this drug. The left atrial pressure also was lower, but this change was not significant statistically. There was a decreased cardiac output at the time of the greatest hexamethonium effect. The total peripheral resistance remained unchanged. The average total pulmonary resistance did not decrease, and the decrease in the pulmonary arteriolar resistance was small. Neither of these two latter

TABLE IV. MEAN VALUES OF HEMODYNAMIC DATA IN FIVE PATIENTS WITH PULMONARY HYPERTENSION IN GROUP II

Conditions	State	B.A.P. (mm. Hg) S/D	B.A.P. (mm. Hg) M	P.A.P. (mm. Hg) S/D	P.A.P. (mm. Hg) M	Pul. Cap. Press. (mm. Hg)	C.I. (L./min./M.²)	Total Pul. Resist. (A.U.*)	Pul. Arteriolar Resist. (A.U.)	Total Periph. Resist. (A.U.)	Ht. Rate (beats/min.)	S.I. (c.c./min./M.²)	Wk. R.V. (Kg. m./min./M.²)	Wk. L.V. (Kg. m./min./M.²)	Br. A. O_2 Sat. (%)	P.A. O_2 Sat. (%)	O_2 Consump. (c.c./min.)
	Rest	122/71	88	65/36	45	24	2.42	835	410	1,611	78	32.5	1.5	2.9	90.3	62.7	240
At rest and during exercise, before hex. admin.	Exercise	151/84	106	100/55	75	34	3.49	977	556	1,541	107	33.9	3.5	5.1	89.8	39.5	645
	Statist.† Significance	t = 3.32 p = 0.05		t = 11.9 p = 0.01		t = 4.27 p = 0.05	t = 6.3 p = 0.01	t = 3.73 p = 0.05	t = 3.40 p = 0.05	t = 0.09 N.S.	t = 7.1 p = 0.01						
	Per cent Change	+24/+18	+17	+54/+53	+67	+42	+42	+15	+36	-4	+37	+4	+133	+76	-0.6	-37	
	Rest	101/66	77	46/28	35	19	2.55	666	340	1,374	100	25.8	1.1	2.6	89.9	62.0	252
At rest and during exercise, after hex. admin. (av. dose = 46 mg.)	Exercise	111/70	84	82/49	60	31	2.91	928	466	1,280	119	25.0	2.3	3.3	90.1	42.0	537
	Statist.† Significance	t = 1.78 N.S.		t = 4.48 p = 0.05		t = 4.44 p = 0.05	t = 1.5 N.S.	t = 2.4 p = sl. <0.05	t = 1.21 N.S.	t = 0.6 N.S.	t = 3.61 p = 0.05						
	Per cent Change	+10/+6	+9	+78/+71	+71	+63	+14	+40	+37	+7	+19	+3	+110	+27	+0.7	-32	
	Exercise	151/84	106	100/55	75	34	3.49	977	556	1,541	107	33.9	3.5	5.1	89.8	39.5	645
During exercise before and after hex. admin. (av. dose = 46 mg.)	Exercise + Hex.	111/70	84	82/49	60	31	2.91	928	466	1,280	119	25.0	2.3	3.3	90.1	42.0	537
	Statist.† Significance	t = 3.16 p = 0.05		t = 5.24 p = 0.01		t = 2.12 N.S.	t = 2.82 p = 0.05	t = 0.94 N.S.	t = 0.45 N.S.	t = 0.57 N.S.	t = 2.54 N.S.						
	Per cent Change	-26/-17	-21	-18/-11	-20	-9	-17	-5	-18	-17	+11	-26	-34	-35	+0.3	+6.0	

*Absolute units. For key to abbreviations see footnote to Table II. †Pertains to the absolute values, and not to the per cent changes.

changes was significant statistically. The right and left ventricular work output decreased during exercise following the administration of hexamethonium by about the same amount (34 per cent).

Summary: Prior to hexamethonium administration, exercise resulted in a slight increase in the brachial artery pressure, and in a considerable increase in the pulmonary artery and left atrial pressures. The cardiac output increased by 42 per cent. The TPR remained constant, but there was a definite increase in the total pulmonary resistance (15 per cent) and a greater increase in the pulmonary arteriolar resistance (36 per cent).

Exercise after hexamethonium administration caused an insignificant rise in the brachial artery pressure when compared to this pressure at rest with hexamethonium. There was still a marked increase in the pulmonary artery and left atrial pressures. The cardiac output increased only slightly (14 per cent). The TPR remained constant as before, but there was still a considerable, and now equal, increase in the total pulmonary and pulmonary arteriolar resistances (40 per cent). The increase in the work of the right ventricle was much greater than that of the left ventricle, as it also was prior to hexamethonium administration.

DISCUSSION

The administration of hexamethonium to these patients with pulmonary hypertension while at rest and supine resulted in a decrease in the average mean brachial and pulmonary artery pressures that was approximately equal (Table II). The cardiac output remained constant, presumably due in part to the slight increase (15 per cent) in heart rate.

Two physiological mechanisms may have been effective in the lowering of the pulmonary artery pressure. These are: (1) a redistribution of blood, with a shift from the pulmonary to the systemic circuit; (2) the inhibition of pulmonary "arteriolar" vasoconstriction.

The decrease in pulmonary artery pressure may have been due in part to a redistribution of blood with a shift from the pulmonary to the systemic circuit which resulted from the decrease in total peripheral resistance effected by hexamethonium. That this could have occurred has been demonstrated by Werkö and associates[11] as well as by Sarnoff and his co-workers.[12, 13] The latter group stated that when the left atrial pressure was high, the removal of only a small volume of blood caused a striking decrease in pulmonary artery pressure, and that peripheral vasodilation and a systemic pooling of blood could account for this result.

Evidence suggesting that the decrease in pulmonary artery pressure also was effected in part by an inhibition of pulmonary "arteriolar" vasoconstriction by hexamethonium resulted from this study. The percentage decreases in total pulmonary and total peripheral resistance were almost identical. However, the percentage decrease in the average pulmonary "arteriolar" resistance was greater (Table II), indicating that hexamethonium resulted in pulmonary "arteriolar" dilatation with a decrease in the pressure gradient

between the pulmonary artery and the left atrium. That the pulmonary vasodilatory response was not more striking might have been the result of pathologic changes in the pulmonary vasculature in mitral stenosis which are frequently present, and thus the response of these vessels might have been somewhat impaired. It must also be considered that the autonomic nervous system might assume a more effective role in the causation of increased pulmonary arteriolar resistance only with higher levels of left atrial pressure, and before marked pathologic changes have taken place in the walls of the blood vessels of the lungs.

That pulmonary "arteriolar" vasoconstriction can be a major factor in the etiology of pulmonary hypertension and that hexamethonium does cause pulmonary "arteriolar" vasodilatation was suggested by the results obtained in Patient 8 (I. B.) who had lupus erythematosus. The administration of hexamethonium resulted in a decrease in the pulmonary arterial pressure of over 50 per cent, while the left atrial pressure and the cardiac output remained constant. The fall in systemic blood pressure was slight, minimizing the effect of any possible decrease in the pulmonary blood volume. This striking decrease in pulmonary arteriolar resistance suggested that the relatively small dose of hexamethonium (25 mg.) resulted in an effective inhibition of the autonomic nervous control of the blool vessels of the lungs in this patient with resultant pulmonary vasodilatation. A lung biopsy done soon after the first cardiac catheterization showed thickening of the alveolar septa by a moderate amount of fibrous tissue and an irregular thickening of the arteriolar walls with some hyalinization of the intima. Despite these pathologic changes, a comparable decrease in pulmonary arteriolar resistance occurred again after giving 25 mg. of hexamethonium at the time of a second study 6 weeks later.

The decrease in the resistance of the pulmonary vasculature (pulmonary arteriolar resistance) following the administration of hexamethonium at rest suggests that a decrease in the neurogenic arteriolar tone occurred. A decrease in the pulmonary arteriolar resistance in patients with mitral stenosis has also been demonstrated by Fowler[2] and by Scott[14] employing tetraethyl ammonium chloride and by Judson and co-workers[4] using hexamethonium. The latter came to the final conclusion that hexamethonium causes changes in the pulmonary circulation secondary to its systemic effects. However, Halmàgyi[18] attributed decreases in pulmonary vascular resistance in patients with mitral stenosis and left heart failure to the inhibition of neurogenic vasoconstrictor tone.

The average cardiac output during exercise was less after hexamethonium administration than during exercise prior to giving this drug (Table IV). Therefore, although the systemic and pulmonary artery pressures had decreased, the systemic, total pulmonary, and pulmonary arteriolar resistances were not significantly altered. A good portion of the small decrease in right ventricular work during exercise was also apparently due to the decreased cardiac output after hexamethonium administration. This finding would limit the usefulness of this drug in chronic pulmonary hypertension.[9] It still is

worthy of trial in acute pulmonary hypertension when the patient is at rest and supine, as in pulmonary embolism and acute pulmonary edema. Under these conditions the decrease in total pulmonary and pulmonary arteriolar resistance observed after hexamethonium administration would be desirable.

It is apparent that the increase in pulmonary arteriolar resistance with exercise is not neurogenically mediated since it is approximately equal before and after hexamethonium block. Prior to giving hexamethonium, exercise resulted in an increase of cardiac output of 42 per cent as compared to that present during the resting state. Following the administration of this drug, exercise resulted in only a 14 per cent increase in the cardiac output (venous return). This relative decrease in venous return during exercise (Tables III and IV) was presumably due to the pooling of blood in the systemic circuit.[8] Yet, exercise after hexamethonium resulted in a considerable elevation of the average pulmonary artery and left atrial pressures in all 5 patients to a level comparable to that present during exercise prior to giving this drug. Therefore, a considerable and almost equal increase in total pulmonary and pulmonary arteriolar resistances occurred. The factors and mechanisms effecting this increase are not entirely clear. It may be hypothesized that a shifting of blood from the pulmonary to the systemic circuit had taken place in the resting state after hexamethonium had been given, resulting in a decreased cross-sectional area of the pulmonary vascular bed and perhaps even a decrease in size of the left atrium.[12, 13] Resistance to flow may vary inversely with the level of pressure due to larger vessel diameters at higher pressures than at lower ones.[16] It might also be postulated that a "critical closing pressure" exists for the pulmonary vessels so that, when the mean pulmonary artery pressure is lowered to a level near the left atrial pressure, a decrease occurs in the number of parallel vascular pathways through the lungs which are perfused with blood. Evidence in favor of this hypothesis has been presented recently.[17] These mechanisms could have resulted in an increased resistance to the slight but sudden increase in blood flow which occurred during exercise after hexamethonium.

SUMMARY AND CONCLUSIONS

1. The administration of hexamethonium chloride via cardiac catheter to 15 patients with pulmonary hypertension at rest and supine resulted in a lowering of the brachial artery, pulmonary artery, and left atrial pressures; the cardiac output remained constant.

2. The pulmonary arteriolar resistance decreased to a greater extent than either the total pulmonary or total peripheral resistances, suggesting the inhibition of pulmonary arteriolar vasoconstriction by hexamethonium.

3. A second factor which may have been effective in lowering the pulmonary arterial pressure was a shifting of blood from the pulmonary to the systemic circuit as a result of the decrease in the total peripheral resistance.

4. The marked increase in pulmonary artery pressure during exercise after hexamethonium administration may be related to the decreased volume

and relative inelasticity of the pulmonary vascular bed but also to a closure of vascular pathways in the lungs, resulting in a greater resistance.

5. The decrease in cardiac output during exercise after hexamethonium administration as compared to the level before the giving of this drug would appear to limit its use in chronic pulmonary hypertension. It might be worthy of trial in acute pulmonary hypertension.

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EVALUATION OF MECAMYLAMINE IN THE TREATMENT OF HYPERTENSION

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ALTHOUGH ganglionic blocking agents have a definite field of usefulness in the management of patients having severe hypertension, their application has been limited by the side effects due to parasympathetic blockade,[1] by their variable absorption by the oral route,[2] and by the depressing effect on general well-being which is apparently induced by their primary effect of producing an orthostatic blood pressure reduction; the latter being chiefly dependent on a reduction in cardiac output.[3, 4] The advent of mecamylamine, a ganglionic blocking agent which does not have the quaternary amine structure and which is totally absorbed by the oral route, led us to study this agent in order to evaluate its effectiveness, acceptability, and important side effects or complications, and to compare its hemodynamic effects with those of other clinically useful ganglionic blocking agents.

METHODS

Every patient who remained on mecamylamine therapy more than 3 months has been included in this study. There was a total of 31 patients. Treatment was usually instituted at a dosage of 2.5 mg. the first day, 2.5 mg. twice on the second day, and 2.5 mg. 3 times on the third day. Gradual dose increments of 2.5 mg. 2 or 3 times daily were given during the following days or weeks until the standing blood pressure recorded in the clinic or by the patient at home fell to 150/90 mm. Hg or until the patient could not tolerate the side effects due to parasympathetic blockade. The total daily sustaining dose, which was reached 2 to 4 weeks after starting therapy, usually ranged between 10 and 90 mg., preferably divided into 3 daily doses. The average daily dose varied between 25 and 30 mg.

Sensitivity to the depressor effects of the medication was essentially unpredictable. Because it was seen in one splanchnicectomized and in one uremic patient early in the study, the starting dose was reduced in similar patients to 1 mg. twice daily. However, in 4 of 7 sympathectomized subjects the maintenance dose was less than the average; in 3 of the 4 azotemic subjects (NPN 45 to 60 mg. per cent) the daily dose exceeded the average.

Home blood pressures were taken in the standing position before the 8 A.M. and 6 P.M. dose of medication. No other hypotensive agent was used. The patients were ambu-

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Read by title at the meeting of the Central Society for Clinical Research, October, 1955.

Supported in part by grant H 1715 from the National Heart Institute, National Institutes of Health, Department of Health, Education and Welfare.

We are indebted to John R. Beem, M.D., Merck, Sharp & Dohme Division of Merck & Co., Inc., for the supply of mecamylamine (Inversine) used in this study.

Received for publication Jan. 21, 1957.

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latory and ranged in age from 26 to 56. Treatment with mecamylamine was not instituted unless the diastolic blood pressure as determined in the clinic after 30 minutes' rest was higher than 110 mm. Hg. Only 3 patients had pretreatment systolic pressures below 200 mm. Hg.

RESULTS

Effect on Standing Blood Pressure.—Fig. 1 presents the response of the standing blood pressure to mecamylamine administration. The individual blood pressure means of the first 2 weeks of treatment were chosen because the number of readings prior to treatment was not sufficient in all cases for purposes of comparison. Each mean was calculated by averaging the daily morning and evening blood pressure values recorded at home. It can be seen that 50 per cent of the patients exhibited a significant drop of the standing blood pressure. Fig. 2 shows the distribution of the blood pressure response during therapy in 20 patients having complete home records throughout the

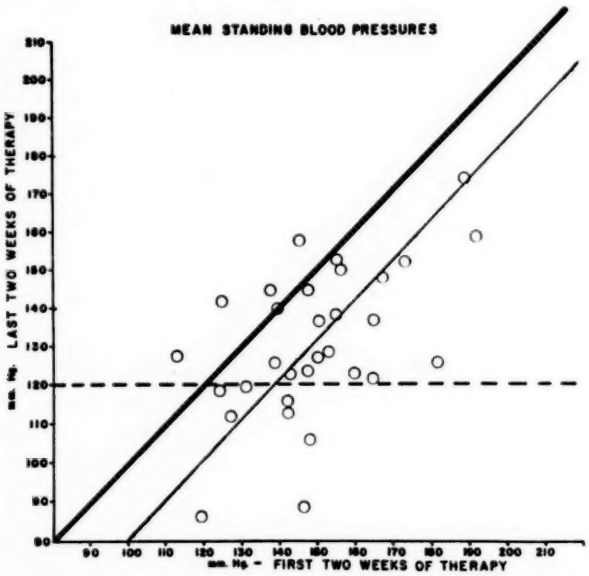

Fig. 1.—The mean values of standing blood pressure during the first 2 weeks of therapy are plotted against those during the last 2 weeks of therapy for each patient. The horizontal interrupted line represents an arbitrary upper limit of mean normal pressure on the scale of the last 2 weeks of therapy. Points lying on the diagonal heavy line represent no change in mean blood pressure, above the line an increase, and below the line a decrease with therapy. Points below the thin diagonal line represent reductions of mean standing blood pressure in excess of 20 mm. Hg.

3 months of observation. The classifications of response to therapy were done for each individual and for each week separately. Using this method of evaluation, some individuals changed their degree of response from one week to the other.

It is evident from Fig. 2 that the number of individuals showing good and fair responses increased during the first 4 weeks from 35 to 59 per cent. This increment grossly paralleled the increase of the mean mecamylamine dose. With a relatively constant mean maintenance dose ranging from 164 to 172 mg. per week, the per cent of individuals having fair and good responses fluctuated between 59 and 68 per cent between the fifth and eighth weeks.

The per cent showing a good response during this period ranged between 18 and 33. The decrease in this per cent having fair and good responses to 44 during the ninth and tenth weeks of treatment is hardly significant. The mean weekly dose had to be gradually increased to 200 mg. in the twelfth week in order to reach the previous percentage of good and fair responses.

Fig. 2 also demonstrates that a fair or good response had occurred in some patients during the first 2 weeks of therapy. Therefore, the blood pressure means of the first 2 weeks, which were compared in Fig. 1 with the blood pressure means of a later stage of treatment, are not true controls as there has already been some lowering of the blood pressure at this time. Utilizing these criteria reduces the number showing good and fair responses. After

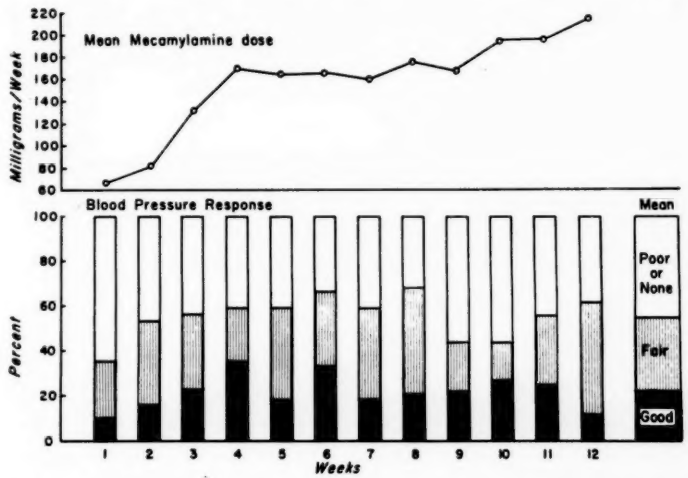

Fig. 2.—The per cent of patients showing a fall in standing blood pressure (below) and the mean dose of mecamylamine administered each week during therapy (above). ■ represents a good response with the standing blood pressure below 150/90 mm. Hg. ▥ represents a fair response with the fall in the mean standing blood pressure being greater than 20 per cent of the mean control pressure but the levels being above 150/90 mm. Hg. □ represents less than 20 per cent reduction from the mean control value.

the first 4 weeks of therapy the incidence of good or fair responses was not related to the dosage of mecamylamine. This may have been in part the result of inability to increase dosage in some individuals because of side effects.

More careful analysis of apparent "failures" indicates that many are a result of the criteria used. In some of the 9 cases in Fig. 1 having the poorest results, other considerations entered into the failure to reduce the standing blood pressure. In 3 cases there were no home blood pressure records. For example, in the case of D. Ro. a small dose of 4.5 mg. per day was given and appeared effective in reducing the mean blood pressure in the clinic from 145 to 117. Subsequently the clinic blood pressure rose to 130, 151, and 148, but the dose was not increased because of the complaint of orthostatic dizziness at home. In 3 cases use of the blood pressure during the first week of therapy resulted in "control" readings which already reflected therapeutic effect. For example, J. Ai. was admitted to the hospital because of apparent focal encephalopathic episodes and responded immediately to 5 mg. daily. His "control" blood pressure for the first 2 weeks was 122. When the cause of his

encephalopathy was found to be unrelated to hypertension, a final treatment blood pressure of 138/100 (119 mean) was considered satisfactory, this being a 50 mm. systolic decrement from the recumbent level. There were 3 clear failures: In one drug induced reductions in pressure caused chest pain; one patient was miserable from gastrointestinal side effects; and in the last patient blood pressure was so variable that sympathectomy was recommended.

Perhaps the degree of blood pressure control achieved can best be demonstrated by the effect of drug withdrawal. Eight patients under satisfactory control were asked to withhold treatment for a period of 1 to 2 weeks. Table I presents these observations. It may be seen that a significant depressor effect was evident in all cases and that the effective dosage had not greatly changed when control was re-established. This indicates lack of tolerance to the medication, since in comparable experiments with quaternary blocking agents a considerable sensitivity to this dosage would have been manifest after such a period of time off therapy.

TABLE I. EFFECT OF TREATMENT WITHDRAWAL ON STANDING BLOOD PRESSURE

PATIENT	STANDING BLOOD PRESSURE (MM. Hg) RELATED TO TREATMENT WITHDRAWAL			DRUG DOSE (MG. PER DAY)	
	BEFORE	DURING	AFTER	BEFORE	AFTER
D. W.	144/ 98	166/122	146/106	60	60
A. C.	140/104*	201/143*	164/128	50	50
W. S.	166/118	215/158	132/ 98	75	60
E. R.	120/ 85*	180/132*	100/ 70*	30	35
L. M.	128/ 90	205/134	152/ 94	45	36
P. R.	125/ 98	250/160	150/115	67	75
L. T.	----	178/128	179/ 95	60	60
L. W.	148/ 92	170/130	----	30	--

*Indicates home blood pressure. All readings otherwise represent the average of one or two casual readings in the clinic on the final day of the period indicated.

Effect on Recumbent Blood Pressure.—Except for one patient among the 31 studied, the effect of mecamylamine on the recumbent blood pressure was negligible.

Discrepancy Between Home and Office Blood Pressure Readings.—It was of interest to compare home and office readings for 25 patients. The mean of the standing blood pressure values recorded the day prior to the clinic visit was compared with the mean clinic standing blood pressure. In 4 instances the home readings significantly exceeded the office readings. In 8 cases the clinic values were significantly higher than the home values and in 13 cases home and office readings did not differ. The reliability of the patient's blood pressure readings was tested by a nurse with the patient taking the blood pressure at the same time and under identical conditions. These showed a satisfactory correspondence. These data confirm the validity of utilizing blood pressure recordings taken in the home in the management of patients having hypertension.[5, 6]

Effects on Renal Function.—Inulin and para-aminohippurate clearance studies with an indwelling bladder catheter were performed in the morning on patients in the recumbent position. Three weeks prior to the clearance the patients were placed on a low salt intake, consisting of a diet to which no

salt was added at the table or in the kitchen. Two grams of sodium chloride were added to this regime daily by the patient. The data presented are the mean of 3 periods, each with a length of fifteen minutes (Table II). It will be seen that, in general, treatment slightly decreased filtration fraction, para-aminohippurate and inulin clearance in about half of the individuals studied. Usually such changes were associated with a decrease in urine flow and in sodium and chloride clearance, but no distinct change in potassium excretion or nonprotein nitrogen.

These changes were then analyzed with respect to the success of antihypertensive therapy in the interval between the two tests. Three patients (W. L., T. L., and S. W.) whose home standing blood pressures did not change significantly showed a decrease in glomerular filtration rate ranging from 21 to 24 per cent. Three patients (C. A., R. E., and M. L.) having a marked drop of standing blood pressure for a prolonged period of time showed no change in renal plasma flow. The glomerular filtration rate decreased in two patients and remained unchanged in one. It is interesting to note that these patients who had a significant response to therapy had impairment of their renal function prior to treatment.

Effects on Cardiac Output and Cardiac Status.—Symptoms of left ventricular failure, which occurred in 8 of the 31 patients, were relieved during the period of study. Angina pectoris became worse in 4 and disappeared in 1 individual. In our further experience to date with 80 patients treated for variable periods of time mecamylamine did not appear to reduce the incidence of angina or myocardial infarction. Indeed in one patient whose standing blood pressure dropped to normal values during treatment with mecamylamine, an anteroseptal myocardial infarction occurred. In one other case a lethal myocardial infarction occurred one week after the hypotensive therapy was stopped. A third patient exhibited electrocardiographic changes in the course of therapy which were suggestive of a subendocardial infarction. In a fourth patient (W. W.) similar electrocardiographic changes appeared after 4 days of therapy. On the basis of these data, it cannot be decided whether this relatively high incidence of myocardial infarction (5 per cent) seen in the total number of treated patients (80) is coincidence or whether mecamylamine did more in promoting coronary occlusion than would any hypotensive agent.

The lesser frequency of extreme weakness and fatigue with this drug suggested that perhaps it did not decrease cardiac output as other ganglionic blocking agents have been shown to do.[3, 4] Accordingly, through use of the tracers, radioactive iodinated serum albumin, or fluorescein, cardiac output and stroke volumes were measured in the sitting position before and during therapy with mecamylamine in 3 individuals. One patient who received an effective dose of 60 mg. on the second and third days showed a marked decrease (45 per cent) of the cardiac output on the third day. However, the high initial cardiac output in this patient was perhaps the result of anxiety and the subsequent reduction might not be entirely attributable to drug

TABLE II. RENAL FUNCTION STUDIES BEFORE AND DURING MECAMYLAMINE THERAPY

PATIENT	TREATMENT DURATION (DAYS)	ARTERIAL BLOOD PRESSURE (MM. Hg) RECUMBENT	STANDING	C_{In} (ML./MIN.)	C_{PAH} (ML./MIN.)	FILTRATION FRACTION	URINE VOLUME (ML./MIN.)	C_{Na} (MEQ./MIN.)	C_{Cl} (MEQ./MIN.)	C_{K} (MEQ./MIN.)	BLOOD NPN (MG. %)
		216/122	224/132	46	124	0.357	1.84	1.3	4.5	12.5	73
M. L.	77	245/128	117/ 81	26	119	0.222	3.55	3.2	3.6	12.9	65
		175/115	182/132	75	367	0.205	5.45	6.2	7.0	33.1	--
S. D.	59	150/112	146/112	77	366	0.210	3.09	3.0	3.5	20.1	--
		215/128	236/154	93	364	0.257	5.80	4.7	5.3	37.3	40
C. A.	49	213/135	142/100	68	343	0.198	1.10	0.7	2.4	11.3	--
		160/120	150/120	138	575	0.240	3.78	4.9	5.4	18.0	30
W. L.	126	146/105	129/101	107	522	0.205	2.50	3.3	4.2	30.0	--
		200/136	202/142	85	359	0.239	4.75	4.3	9.7	20.2	35
S. W.	86	187/124	152/116	65	305	0.213	1.43	2.5	3.2	21.0	38
		190/125	160/130	44	196	0.225	1.49	0.6	4.0	14.0	48
R. E.	71	169/115	118/ 78	47	195	0.241	1.23	0.7	0.7	10.1	37
		185/111	184/112	117	558	0.210	4.20	3.4	3.8	44.7	37
T. L.	162	172/108	149/ 97	93	343	0.270	1.75	0.9	3.5	25.1	41
		232/136	234/144	65	266	0.245	2.93	2.4	2.2	20.0	38
W. D.	81	219/132	158/112	70	261	0.268	4.82	1.7	1.6	18.8	--
		229/159	230/140	60	250	0.241	1.34	1.4	1.4	4.5	35
R. P.	80	198/149	168/132	--	201	----	1.58	1.2	1.2	7.1	46

For each patient the upper row of values was obtained before and the lower row during mecamylamine therapy.

therapy. Two other patients received smaller doses and their sitting blood pressures as well as their cardiac outputs on the seventh and eighth days, respectively, of treatment did not change significantly.

Effect on Blood Coagulation.—Because of the occurrence of coronary thrombosis during therapy a study of blood coagulation factors was undertaken. Studies of prothrombin time, fibrinogen, and factors V and VII revealed no changes in 5 patients studied over a period up to 8 months in duration.[7]

Effect on Number of White Blood Cells and Hemoglobin Concentration.—There were no alterations found over a treatment interval of 2 to 4 months in 10 patients studied.

Side Effects and Complications of Treatment.—The usual effects of parasympathetic blockade were observed (blurred vision, dryness of the mouth, constipation, and impotence) in most patients who received an effective dose. Constipation was the most prominent side effect. Patients who had been on chlorisondamine often noted fewer ocular symptoms when their blood pressure had been reduced to equivalent levels by mecamylamine. The majority of patients reported fewer attacks of dizziness or weakness than when taking pentolinium. Constipation, dry mouth, and impotence were as frequently noted with mecamylamine as with other ganglioplegic drugs.

Ten of the patients complained of some loss of muscular strength. One patient developed ileus and volvulus while on a small therapeutic dose of 5 mg. daily. An unusual side effect noted by 2 patients was extreme chilliness associated with a tremor. A confused mental state developed in 2 individuals. Such production of tremor and mental confusion has been noticed as well by others. This is sometimes related to impaired renal function, although as noted above we have treated 4 chronically azotemic individuals without difficulty. In all instances the effects have cleared on withdrawing therapy. In Table III are summarized the side effects among the present consecutive series and in our subsequent experience to date (80 cases in all).

Effects on the Retinal and Cerebral Circulation.—In a few patients fresh retinal hemorrhages and exudates appeared to clear during prolonged therapy with mecamylamine. Cerebrovascular episodes have not recurred during the 60 patient-months of treatment in 5 subjects who before treatment had had a cerebrovascular accident. Unpublished observations[8] would indicate that 30 per cent of the hypertensive patients suffering one focal neurologic episode will have a recurrence within 5 years. Our experience is, therefore, inadequate to draw satisfactory conclusions concerning the prevention of this complication.

DISCUSSION

Mecamylamine has proved to be a useful ganglionic blocking drug for the management of severe hypertension. The frequency of orthostatic blood pressure reduction achieved in this series of 31 patients over a 3-month interval is comparable to that observed with quaternary blocking agents. Twenty-three per cent of the patients showed a good blood pressure response, their

TABLE III. REASONS FOR ADVISING CESSATION OF MECAMYLAMINE THERAPY

CASE INITIALS	TREATMENT DURATION	TOTAL DAILY DOSE (MG.)	UNACCEPTABLE SIDE EFFECTS	
			INTRACTABLE GI SIDE EFFECTS	OTHER CAUSES
1. E. Kl.	2 mo.	20		Also had adequate control on reserpine alone
2. M. Cl.	2 mo.	40	Yes	Poor blood pressure response
3. C. Ro.	4 mo.	50	Yes	Poor blood pressure control
4. M. Po.	4 mo.	25	Yes	Poor blood pressure control
5. K. La.	3 mo.	30	Yes	Poor blood pressure control
6. F. Ro.*	2 mo.	6	Yes	Increased angina at same blood pressure reduction as achieved by other ganglion blockers
7. C. Br.	5 mo.	45	Yes	Constant anterior chest pain. Poor blood pressure control
8. E. Tu.	1½ mo.	30	Yes	
9. M. Bu.	2 days	2.5	Yes	60-year-old woman suddenly developed a marked ileus; at operation volvulus found
10. F. Fa.*	3 days	5		Cerebrovascular episode on third day
11. J. Vo.	3 mo.	18		Malignant hypertension with rapid terminal azotemia
12. R. Mo.	2 mo.	35	Yes	Also itching, sore eyelids, "jitters," and dyspnea, recurrent when drug restarted
13. P. Bu.	1 mo.	35		"Jittery," gross tremor, memory loss, and confusion
14. G. Re.*	7 days	3-9		Early uremic symptoms. NPN 113. After 5 days irregular treatment patient developed confusion, aphasia, increased motor activity, and orthopnea. When treatment stopped, he lapsed into uremic stupor

Above cases represent all instances among the last 80 patients placed on mecamylamine treatment in which it has seemed advisable to the clinic physician to omit the drug.

*Indicates coexistence of moderately elevated nonprotein nitrogen.

standing blood pressures falling to normal levels. Thirty-three per cent exhibited a fair response, while in 44 per cent there was an unsatisfactory response. Seven patients had had splanchnicectomies prior to treatment and in 4 cases their maintenance doses were less than the average. That sympathectomy sensitizes the patient to ganglionic blocking agents has been demonstrated in the case of tetraethyl ammonium responses.[9]

Each patient must find that dose which effectively lowers his standing blood pressure. Standing blood pressures taken before breakfast and supper are fairly representative of the total diurnal effect. Readings at these times permit adjustment of individual doses to secure better morning or evening blood pressure control. When dizziness or fatigue at noontime is reported,

blood pressure should be checked, since usually it is lower at this time than in the evening. Medication should be taken at mealtime and increased by 2.5 mg. increments every second or third day until the average standing blood pressure reaches normal values or side effects make further increase impossible.

Although the effect on recumbent blood pressure is negligible, definite cardiac and retinal improvement has occurred in some patients. Renal circulation is unimproved or may be considerably reduced in the upright position,[10] and cardiac output appears to be reduced as with other ganglionic blocking agents. Corcoran and Page[11] have shown that renal plasma flow may improve only after years of therapy with hexamethonium so that transient reductions of renal plasma flow in the early stage of treatment should not discourage one from using a ganglionic blocker. One should make sure that the functional renal reserve is sufficient to stand transient reductions of renal blood flow. Repeated checking of the nonprotein nitrogen or blood urea nitrogen is a necessity in cases with azotemia.

Although the relatively high incidence of coronary thrombosis may be coincidental, it is pertinent to stress the importance of this complication. In other series this condition has not increased with effective therapy.[12] Patients having suffered a myocardial infarction or having signs of coronary insufficiency should be treated very carefully with ganglionic blocking agents and marked falls of their standing blood pressures must be avoided.

Side effects were comparable to those with quaternary ammonium compounds. Weakness appeared to be a less frequent complaint with mecamylamine than with other ganglionic blocking agents. Constipating effects were similar to those following other ganglionic blocking drugs, but since absorption is relatively complete, variation in bowel motility does not affect the amount absorbed. This is a further advantage of this drug. Visual effects were possibly less marked with mecamylamine. Dry mouth and impotence were about as frequent as with quaternary blocking drugs. Tolerance appeared to be negligible. These observations are in general agreement with those of others investigating this form of therapy.[12, 13]

SUMMARY

1. The status of 31 consecutive hypertensive patients treated as outpatients with mecamylamine and followed for over 3 months was examined. A daily record of standing blood pressure showed a significant drop in over 50 per cent. Approximately one-third showed normal daily standing blood pressures. In approximately 20 per cent medication had to be discontinued because of distressing side effects.

2. Chronic administration of the drug produced only a slight reduction in renal plasma flow, glomerular filtration rate, and in sodium clearance in about half the cases.

3. In 2 cases in which there was little change in the sitting blood pressure cardiac output was unaltered. In a third instance a reduction in cardiac output occurred concomitant with a pronounced fall in sitting blood pressure.

4. Symptoms of left ventricular failure were relieved. Angina pectoris was made worse in 4 of 5 cases and myocardial infarctions were observed while on treatment, but this may have been coincidental. No influence of therapy on the blood clotting mechanism was seen.

5. It is concluded that this drug offers the best present means of controlling the orthostatic blood pressure in severe hypertensive disease.

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SIGNIFICANCE OF INCREASED URINARY PEPSINOGEN (UROPEPSIN) EXCRETION DURING ACTH ADMINISTRATION AND IN DUODENAL ULCER PATIENTS

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IN a previous paper on the effects of ACTH gel given for 5 to 6 days to 5 normal subjects,[1] we reported an immediate, steep, and sustained increase in urinary pepsinogen, with a variable, small increase in the concentration and in the output of pepsin by the stomach. In the present report, an analysis is made of the factors involved in the increase of urinary pepsinogen in 5 normal subjects during ACTH administration, and in 6 patients with peptic ulceration. The results indicate that different mechanisms are responsible for the increases of urinary pepsinogen excretion under these two conditions.

METHODS

Five normal male medical students were studied, one on two separate occasions. After a 5- to 8-day control period, 25 clinical units of ACTH gel were given by intramuscular injection every 12 hours for 5 to 6 days, followed by a further 4- to 9-day period of observation. The response of the adrenal cortex was measured by the determination of consecutive, 24-hour 17-hydroxycorticoid excretion in the urine,[2] and of daily total eosinophil counts employing 0.45 per cent phloxine in 50 per cent propylene glycol.

The pepsinogen and creatinine content of consecutive 24-hour urine collections and of plasma collected each morning in the fasting state were determined; the creatinine by the method of Bonsnes and Taussky,[3] and pepsinogen by methods described previously,[4, 5] the only modification being an increase of the incubation time of urine to 30 minutes. One-hour gastric aspiration was performed every second day in the fasting state, and the gastric juice analyzed *inter alia* for pepsin content by the hemoglobin method.[4] Results are expressed in the same units for blood (P.U. per 100 ml.) as for urine (P.U. per hour) and gastric juice (P.U. per hour). The standard deviation for four different quintuplicate plasma pepsinogen estimations was 3 per cent.

In addition to the above studies, the plasma and urine pepsinogen levels of 2 cases of steroid-induced peptic ulcers, both reported in detail elsewhere,[1, 7] and of 4 other ulcer patients with high urinary pepsinogen levels have been included in the results.

RESULTS

The results in the 5 normal subjects receiving ACTH are summarized in the table. All showed an adequate adrenocortical response to ACTH, as judged by an invariable eosinopenia and an increase in 17-hydroxycorticoid excretion. ACTH produced a significant decrease in urine output, followed by diuresis after its discontinuation. Urinary pepsinogen excretion rose sharply on the first day

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This study was supported by grants from the United States Public Health Service (USPHS RG4298 [c]) and from the Horace H. Rackham School of Graduate Studies.
Received for publication Sept. 27, 1956.

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TABLE I. EFFECTS OF I.M. ACTH GEL IN 5 NORMAL SUBJECTS (MEANS ± S.D.)

PATIENT	TIME	NO. DAYS	GASTRIC PEPSIN (P.U./HR.)	EOSINOPHILS (CELLS PER C.MM.)	URINE				PLASMA		CLEARANCE (L./24 HR.)	
					17-HYDROXY-CORTICOID (MG./24 HR.)	VOLUME (L./24 HR.)	CREATININE (GM./24 HR.)	PEP-SINOGEN (P.U./HR.)	CREATININE (MG. %)	PEP-SINOGEN (P.U. %)	CRE-ATININE	PEP-SINOGEN
O. G. W.	Pre-	7	89,000	200 ± 30	0.5 ± 0.8	1.300 ± .283	2.313 ± .125	317 ± 32	1.27 ± .01	14.1 ± 2.4	184 ± 8	57 ± 8
	ACTH	**5**	**86,000**	**18 ± 12**	**23.7 ± 12.5**	**1.030 ± .262**	**2.400 ± .098**	**438 ± 41**	**1.25 ± .08**	**15.2 ± 4.0**	**193 ± 5**	**76 ± 24**
	Post	7	56,000	216 ± 28	1.4 ± 2.0	2.030 ± .289	2.436 ± .156	304 ± 30	1.37 ± .07	16.3 ± 2.0	177 ± 7	45 ± 9
D. B.	Pre-	7	40,000	230 ± 26	0.9 ± 2.2	2.430 ± .977	2.376 ± .10	212 ± 30	1.20 ± .05	11.0 ± 1.4	197 ± 14	49 ± 6
	ACTH	**5**	**36,000**	**2 ± 3**	**57.3 ± 16.5**	**1.370 ± .103**	**2.700 ± .165**	**424 ± 33**	**1.11 ± .08**	**10.4 ± 2.6**	**242 ± 32**	**104 ± 30**
	Post	9	50,000	291 ± 32	1.5 ± 1.7	3.030 ± .880	2.461 ± .134	205 ± 46	1.29 ± .05	14.9 ± 5.1	195 ± 17	37 ± 15
J. D.	Pre-	8	125,000	146 ± 28	1.3 ± 2.0	1.050 ± .254	1.796 ± .14	208 ± 42	1.16 ± .13	11.5 ± 2.2	144 ± 15	43 ± 11
	ACTH	**6**	**156,000**	**55 ± 34**	**13.1 ± 7.4**	**.906 ± .209**	**1.867 ± .15**	**310 ± 33**	**1.05 ± .04**	**15.0 ± 1.4**	**184 ± 9**	**51 ± 3**
	Post	5	112,000	185 ± 17	0.4 ± 0.4	1.960 ± .242	1.950 ± .16	201 ± 54	1.37 ± .07	13.8 ± 0.7	143 ± 7	35 ± 0
A. B.	Pre-	5	66,000	108 ± 48	2.7 ± 0.81	1.930 ± .880	2.382 ± .121	277 ± 34	1.28 ± .12	15.7 ± 1.0	169 ± 3	42 ± 9
	ACTH	**6**	**81,000**	**15 ± 11**	**27.8 ± 11.1**	**1.190 ± .209**	**2.228 ± .241**	**460 ± 58**	–	**15.6 ± 5.0**	–	**71 ± 16**
	Post	4	61,000	84 ± 40	0.2 ± 0.2	2.115 ± .532	2.290 ± .38	272 ± 24	–	15.8 ± 2.7	–	43 ± 8
D. C.	Pre-	5	207,000	224 ± 53	1.4 ± 2.5	1.340 ± .391	1.982 ± .19	340 ± 67	1.24 ± 0	17.7 ± 2.5	147 ± 7	43 ± 7
	ACTH	**6**	**226,000**	**17 ± 12**	**13.2 ± 3.0**	**1.260 ± .242**	**2.150 ± .18**	**523 ± 53**	–	**19.9 ± 2.6**	–	**64 ± 8**
	Post	4	205,000	87 ± 33	0.4 ± 0.2	1.895 ± .601	2.06 ± .09	352 ± 57	–	21.1 ± 1.7	–	40 ± 6

of treatment and remained at that plateau for the 6 days of the ACTH administration. There were increases in the clearances of both creatinine and pepsinogen in all the subjects in whom these measurements were made. The changes in creatinine clearance were accounted for by changes in both the urinary and plasma creatinine, while the increases in pepsinogen clearances were almost entirely due to changes in the urinary pepsinogen (see Fig. 2). The mean values in these 5 subjects for the basal output of gastric pepsin before, during, and after ACTH (Table I) indicate the variable effects of ACTH on gastric pepsin and the independently variable changes in plasma pepsinogen concentration. These contrast with the invariable steep increases in urinary pepsinogen excretion due to ACTH. Fig. 1 shows, furthermore, that there was little if any direct correlation between simultaneous measurements of gastric pepsin and urinary pepsinogen.

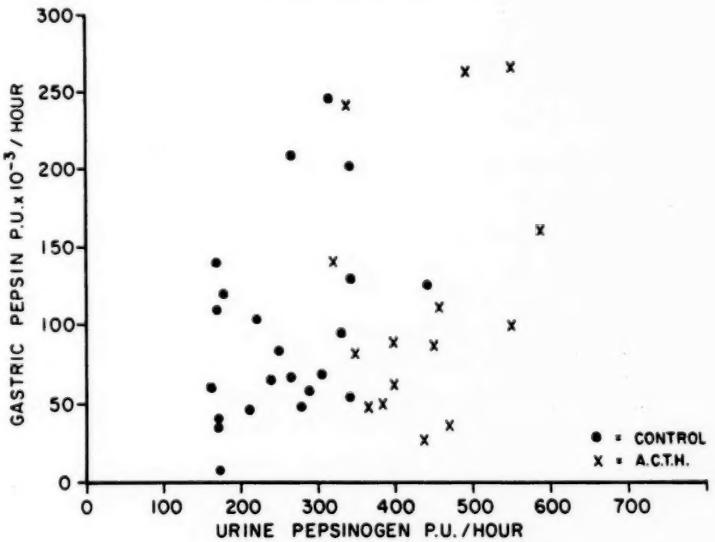

Fig. 1.—Scatter diagram showing poor correlation between gastric pepsin secretion and simultaneous urinary pepsinogen excretion.

Fig. 2 shows the correlation between the level of plasma pepsinogen and the pepsinogen in the urine in the control and ACTH samples in these experiments, and includes 29 samples from 6 patients with peptic ulceration, 2 of whom have been reported elsewhere.[1, 7] The mean clearance of pepsinogen in the control periods in normals was 44 ± 14 liters in 24 hours; in the ulcer subjects, 36 ± 19 liters per 24 hours; and in normal subjects under the influence of ACTH, 65 ± 29 liters per 24 hours, a level significantly higher at the 1 per cent level than either the levels in the control periods or in the ulcer subjects. The increase in pepsinogen clearance, as stated above, resulted from increases in urine pepsinogen, with little change in plasma pepsinogen.

Simultaneous measurements of pepsinogen and creatinine clearances were obtained in the control periods of all the subjects, but in only 3 subjects during and after ACTH administration, and in one subject (Fig. 3, labeled "abnormal

patient'') during a second ACTH study 3 months after the first, when there had been a marked decline in the clearances of both pepsinogen and creatinine. The correlation of the clearances of these 2 substances is shown in Fig. 3 as

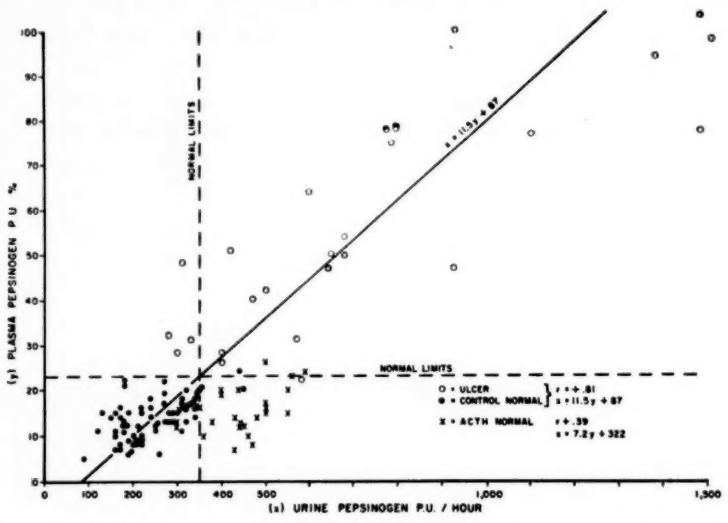

Fig. 2.—Illustration of the different mechanisms responsible for the increased urinary pepsinogen excretion in ulcer patients and in normals given ACTH.

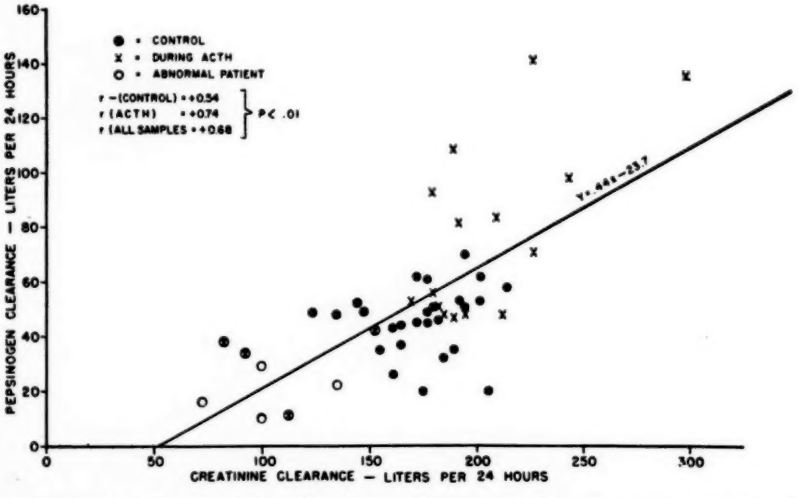
● = CONTROL
X = DURING ACTH
O = ABNORMAL PATIENT
r (CONTROL) = +0.54
r (ACTH) = +0.74
r (ALL SAMPLES = +0.68
P< .01
PEPSINOGEN CLEARANCE — LITERS PER 24 HOURS
CREATININE CLEARANCE — LITERS PER 24 HOURS

Fig. 3.—Relation between creatinine clearances (GFR) and pepsinogen clearances before and during ACTH administration.

being somewhat better during ACTH administration than in the control periods, but both these and the over-all correlation ($r = + 0.68$) are significant at the 1 per cent level.

DISCUSSION

The determination of the renal clearance of any substance requires accurate measurement of the *same* substance in the blood as in the urine. Moreover,

with any extended period of urine collection, the clearance can only be valid if the blood level is determined at frequent intervals or alternatively a single blood level is adequate if there is no variation of any significance in the blood level during the period of urine collection.

To comply with such requirements in measuring endogenous pepsinogen clearances the following facts appear to validate the method as employed in this investigation. First, the proteolytic enzymes active at pH 1.5 to 2.5 in the blood and urine are measured by the same technique and are considered to be only pepsinogen.[5] Second, the blood level of pepsinogen has been shown to remain constant throughout the day and from day to day, irrespective of food intake, dietary composition, activity, or sleep,[14] with a coefficient of variation during the day of 8 per cent.[14] In a group of 13 normal subjects studied by us, and reported elsewhere,[6] the coefficient of variation of the mean plasma pepsinogen concentrations in samples collected every 2 hours for 13 hours was 7 per cent. In a similar group of 13 subjects receiving 25 units ACTH intravenously for 8 of the 13 hours, the coefficient of variation was 9 per cent.

There are always objections to the use of endogenously produced substances such as pepsinogen and creatinine for clearance measurements, since their source of production may be influenced by the conditions imposed to alter their clearance. However, since this study was designed to determine the causes for an increase in urinary pepsinogen occurring naturally, the endogenous substance had to be employed.

The increased excretion of urinary pepsinogen found in the majority of patients with pyloric or duodenal ulcers[4] differs in one important respect from the increased output produced by ACTH or hydrocortisone,[6] viz., that in the former instance it results from an elevated plasma pepsinogen level, while in the latter the plasma levels are normal.

In the usual ulcer subject increases in plasma and urinary pepsinogen probably reflect an increase in peptic cell mass,[5] which appears to be a function of the duration of the disease.[4] On the other hand, an increase in plasma pepsinogen may occur in ulcer subjects as a result of reversal of the pepsinogen secretion gradient, where a striking decline of pepsin secretion *into* the stomach is accompanied by a great increase of the amount of the proenzyme entering the circulation. This phenomenon was observed in the healing stages and for some considerable time after healing of steroid-induced peptic ulceration in 2 subjects reported elsewhere.[1, 7]

Whatever the causes of an elevated plasma pepsinogen, the result is an increased pepsinogen load on the kidney which, with a fixed, normal clearance rate, results in an increased amount of pepsinogen excreted into the urine. With ACTH and hydrocortisone, however, where the plasma pepsinogen levels remain normal, the opposite reason exists for an increase of pepsinogen in the urine, viz., an increased renal clearance of pepsinogen.

The cause or causes of this increased clearance of pepsinogen with ACTH might be sought in one of two renal mechanisms probably responsible for the

normal regulation of pepsinogen excretion. The difference between the average normal pepsinogen clearance of 44 L./24 hours and the glomerular filtration rate (GFR) of about 170 L./24 hours could be accounted for either by some glomerular impediment to the excretion of pepsinogen (molecular weight 42,000),[11] or by tubular reabsorption, or both, as is the case with plasma albumin (molecular weight 47,000).[12] The increases in GFR with ACTH reported here, of the same order of magnitude as those found by others[9, 10] using inulin clearances, and the fair correlation between pepsinogen and creatinine clearances suggest that changes in glomerular filtration play some part in the regulation of pepsinogen excretion. However, the differences between the relative increases in pepsinogen and creatinine clearances (48 and 13 per cent, respectively) suggest that increased glomerular filtration may not account for the whole increase in pepsinogen clearance with ACTH.

Support for tubular reabsorption as a mechanism in the control of urinary pepsinogen excretion may be derived from the increases in urinary pepsinogen caused by phlorhizin.[13] There is, however, no direct evidence at present available to indicate whether either or perhaps both the mechanisms suggested (an increase in the GFR or a decreased tubular reabsorption), is the means whereby ACTH increases the clearance of pepsinogen.

The fact that there is an increase in urinary pepsinogen excretion with ACTH and at the same time no significant fall in the concentration of plasma pepsinogen, implies that the amount of pepsinogen excreted in the urine is being replaced simultaneously into the plasma. This replacement could arise either from the leukocytes, in which there is a considerable amount of pepsinogen in man,[5] or, as seems more likely, from the stomach. In calculating the mean increase in urinary pepsinogen which could be expected during ACTH administration to result purely from the observed elevation of plasma pepsinogen (assuming a constant pepsinogen clearance), one arrives at a figure of only 11 per cent of the observed increase. Therefore, 89 per cent of the increase in urinary pepsinogen excretion resulted from the increase in renal clearance of pepsinogen. The converse calculation for the expected clearance estimated from the observed urinary pepsinogen excretion (assuming a constant plasma pepsinogen level) reveals a discrepancy of 11 per cent between observed and expected clearances during ACTH administration. Thus only 11 per cent of the observed increase in urinary pepsinogen could be ascribed to the elevations observed in plasma pepsinogen, which were small and inconsistent.

These observations suggest that the increase in urinary pepsinogen under the influence of ACTH is due principally to an increased renal clearance of pepsinogen and that the stomach maintains the blood level by some simultaneously acting mechanism which is independent of the secretory function of the stomach. This is confirmed by the lack of correlation between gastric pepsin secretion and the simultaneous measurements of plasma or urinary pepsinogen during ACTH administration.

SUMMARY

ACTH gel given for 5 to 6 days to each of 5 normal subjects caused an immediate and sustained increase of urinary pepsinogen excretion without substantially affecting gastric pepsin output or plasma pepsinogen concentrations. There was, therefore, an increased clearance of pepsinogen by the kidney, a rise of 48 per cent which correlated with relatively smaller increases (13 per cent) in creatinine clearance (GFR). The increased pepsinogen clearance was due to both an increase in GFR and to probable changes in tubular reabsorption of filtered pepsinogen. The absence of a fall in plasma pepsinogen concentration during ACTH administration implied that the amount of pepsinogen lost from the plasma by renal excretion was simultaneously being replaced from the gastric mucosa.

The mechanism whereby ACTH increases urinary pepsinogen excretion differed from that responsible for the high urinary pepsinogen observed in the 6 patients with duodenal or pyloric ulceration. In these patients the high urinary pepsinogen resulted from elevated plasma pepsinogen levels with a normal or even slightly reduced rate of renal clearance of pepsinogen.

We wish to express our thanks to Mrs. Esther Dullaert for her able technical as-

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THE "MUCOPROTEIN FRACTION" (PHOSPHOTUNGSTIC ACID PRECIPITATE) IN TISSUE AND SERUM: A COMPARISON OF NORMAL FINDINGS AND FINDINGS IN LEUKEMIA AND LYMPHOMA

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QUANTITATIVE and, possibly, qualitative changes of one of the fractions of human serum, the phosphotungstic acid precipitate, are found in many diseases and particularly in hematologic disorders of neoplastic nature (lymphomas, leukemias).[1] This fraction has been shown to contain the largest amount of the serum mucoproteins as well as other poorly identified constituents and is generally defined as "mucoprotein fraction." Changes in content and composition of this fraction of serum have been shown to have diagnostic value, but their significance remains obscure. In particular, the available literature does not offer much information as to whether serum changes reflect similar abnormalities in mucoproteins of tissue. The only pertinent experimental observation to be found is by Catchpole,[2] who described parallel changes in glycoprotein content of serum and tissues in a group of mice with transplanted fibrosarcoma. We report in this paper an attempt to correlate content and composition of the phosphotungstic acid precipitate ("mucoprotein fraction") from serum and tissue (spleen) in patients with various hematologic disorders. A significant dissociation of values in serum and tissues was found in patients with lymphoma and leukemia.

MATERIALS AND METHODS

Collection of Specimens.—Serum and splenic tissue were obtained from 27 human beings suffering from a variety of diseases as shown in Table I. Serum was collected and stored as described previously.[1]

Both surgical and autopsy specimens of splenic tissue were studied. *Surgical specimens* were obtained from: (1) 5 patients with advanced lymphoma in whom surgery was performed because of intractable hemolytic anemia or thrombocytopenia with megakaryocytic bone marrow; (2) one patient with chronic malignant reticuloendotheliosis; (3) a miscellaneous group of 9 patients including 3 with acquired hemolytic anemia (idiopathic in one instance, due to fibrous congestion and to brucellosis in the remaining 2), 2 with idiopathic thrombocytopenic

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Received for publication Dec. 28, 1956.

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purpura (proved to be due to scleroderma and sarcoidosis, respectively, at histologic examination), one with aplastic anemia and enormously increased transfusion requirements, one with Cooley's anemia, one with congenital spherocytosis, one who suffered spontaneous rupture of the spleen, site of myeloid metaplasia. *Autopsy specimens* were obtained from 2 patients with lymphoma, 2 with acute leukemia, 5 with miscellaneous diseases (ruptured cerebral aneurysm, Laennec's cirrhosis of liver, brain tumor, chronic glomerulonephritis, and collagen syndrome).

TABLE I. SPLEENS STUDIED IN THE COURSE OF THIS WORK

TYPE	NO.	SURGICAL SPECIMENS	AUTOPSY SPECIMENS
Normal	4	3	1
Lymphoma	7	5	2
Leukemia	3	1	2
Miscellaneous diseases	13	9	4
Total	27	18	9

Surgical specimens were perfused 5 times with cold saline immediately after collection. In 2 instances specimens were washed rapidly with cold saline solution and frozen in liquid nitrogen for optimum preservation of all constituents.[3] Autopsy specimens were obtained within 2 to 4 hours after death. They were promptly washed free of blood with cold saline and immediately transferred to a Deepfreeze at −20° C., until used. It was possible to separate lymphoma tissue from splenic tissue in 2 specimens and, thus, to study "mucoproteins" of the isolated lymphoma tissue. To check the reliability of our results, values obtained for surgical specimens were compared with those for autopsy material specimens in patients of the same disease group. Results were essentially similar. Also, several aliquots of the same spleen were collected with the different techniques mentioned above. Results of chemical analysis were well comparable. Thus, the technique of organ collection did not seem to influence the experimental findings.

Preparation of Tissue for Chemical Analysis.—Frozen tissue was slowly thawed in an icebox at 4° C. for 2 to 6 hours. After thawing, a weighed aliquot of tissue was transferred to a wide test tube containing a volume of cold saline equivalent to 5 times the weight of tissue. Tissue was gently "broken" by squeezing it against the test tube wall by means of a large spatula. Centrifugation at 3,200 r.p.m. at 4° C. for 30 minutes followed; supernatant saline, red with hemoglobin pigment, was discarded. This process was repeated 5 times or until the supernatant fluid no longer contained visible pigment and tissue had assumed a yellowish tinge.* A volume of saline equal to the weight of tissue originally employed was then added. Tissue was mechanically ground in Van Potter's homogenizer for 20 minutes after adding 1 Gm. of sea sand per 10 Gm. of tissue. The ground material was transferred to glass tubes and centrifuged for 1 hour at 4° C., 3,200 r.p.m. per minute. Supernatant yellow fluid, where no intact cells were found, was transferred by aspiration to other test tubes and used for all studies.

Chemical Analyses of Tissue.—These included the determination of total protein (T.P.), by a quantitative biuret technique[4]; the determination of total protein-bound hexoses (T.Ph.), by the tryptophan method[5]; the determination of total hexosamine (T.Ha.), with Rimington's method[6]; and the determination of total cholesterol (T.Chol.), with a modified Zlatkis technique. One-half milliliter of homogenized tissue was added to 10 ml. of a boiling mixture of 1:1 acetone and alcohol. The mixture was filtered and total cholesterol was determined on the filtrate by the method of Zlatkis.[7] Total protein in the phosphotungstic acid precipitate (Mp.), was determined as described by Winzler et al.[8] As already mentioned, the phosphotungstic acid precipitate contains mucoproteins as well as other poorly identified constituents, although it is generally described as "mucoprotein fraction." Homogenized tissue suspended 1:1 in saline solution or serum was used. The following constituents were determined in

*Wright stained smears of the partially homogenized splenic tissue showed fairly well-preserved lymphocytes and lymphoblasts. A mixture of lymphoblasts, reticulum cells, leukocytes, and giant cells was observed in the homogenized lymphoma tissue.

the precipitated fraction: (1) hexoses (Mh.), by the tryptophan method described by Shethlar[6]; (2) hexosamine (Mha.), by an application of Remington's method for serum[6]; (3) cholesterol (Mchol.), as described elsewhere.[9] Since tissue had originally been diluted to 50 per cent with saline all values obtained for tissue were multiplied 2 times to express absolute values.

Chemical Analyses of Serum.—These were carried out with the techniques previously described.[1, 3, 19]

Electrophoresis of Tissue Homogenates.—One-tenth milliliter of tissue homogenate was transferred to a glass slide (1″ × 3″) and the slide so held that the fluid was uniformly distributed along its edge. The slide was then gently applied to a wet paper strip. This technique allowed fairly uniform distribution of the tissue homogenate on the paper strip. Migration was carried out by Durrum's method for serum proteins,[10] lipoproteins,[10] and glycoproteins.[11]

RESULTS

Normal Spleens (Table II).—A preliminary search of the available literature failed to find any data regarding concentration in normal splenic tissue of the chemical components considered in this article. Three surgical spleens and one autopsy spleen were then analyzed to obtain baseline values to interpret pathologic results. Tissue proteins appeared relatively rich in cholesterol and in total hexosamine as compared to serum.[1] Also the ratio of phosphotungstic acid precipitable protein to total protein was higher in normal splenic tissue than in normal serum.*

TABLE II. VARIOUS CONSTITUENTS OF NORMAL SPLENIC TISSUE

CASE NO.	DIAGNOSIS	T.P. (MG.%)	PROTEIN FRACTION T.PH. (MG.%)	T.HA. (MG.%)	T.CHOL. (MG.%)	"MUCOPROTEIN FRACTION"* MP. (MG.%)	MH. (MG.%)	MHA. (MG.%)	MCHOL. (MG.%)
1	Normal	3,600	63	150	106	126	6.0	14.7	2.70
2	Normal	4,200	93	90	140	96	6.0	10.2	1.80
3	Normal	4,800	30	72	200	80	13.2	12.6	1.50
23	Cerebral hemorrhage	3,600	58	96	110	158	4.4	10.0	1.00
24	Chronic glomerulonephritis	2,000	38	90	130	94	8.4	10.5	1.65
25	Myocarditis	3,600	58	120	190	130	5.0	6.6	1.40
26	Cirrhosis of liver	3,300	45	120	130	108	9.3	9.3	2.25
	Average	3,585	55	105.4	143.7	113.1	7.47	10.55	1.76

For key to abbreviations see text.
*Phosphotungstic acid precipitate.

Lymphoma Spleens (Table III).—Seven spleens were analyzed. It was possible in 2 instances to separate and analyze well-delineated, easily separable lymphoma nodules. Lymphoma spleen was richer in total protein than normal tissue. Total protein-bound hexoses and total hexosamine were also increased, in some quantitative relationship to the rise in total protein. Finally, the proteins of lymphoma tissue were rich in cholesterol. Phosphotungstic acid precipitable protein was also increased and relatively more so than total proteins. This fraction, however, appeared relatively poor in hexoses and hexosamine, while the amount of cholesterol showed slight relative increase.

*Electrophoretic studies may have helped in interpreting the high content of phosphotungstic acid precipitable protein in splenic tissue. Electrophoretic strips obtained from normal splenic tissue showed absence of albumin. Since albumin represents a large part of the total protein, its absence could help to explain the low total protein in splenic tissue as compared to serum. On the other hand, splenic tissue is rich in alpha-2 and especially beta-globulin fractions which seem to contain or carry most of the protein moiety precipitable with phosphotungstic acid. The latter fraction should then be increased in splenic tissue (Fig. 1).

TABLE III. VARIOUS CONSTITUENTS OF SPLENIC AND ISOLATED TUMOR TISSUE IN LYMPHOMA

CASE NO.	PROTEIN FRACTION				"MUCOPROTEIN FRACTION"*			
	T.P. (MG.%)	T.PH. (MG.%)	T.HA. (MG.%)	T.CHOL. (MG.%)	MP. (MG.%)	MH. (MG.%)	MHA. (MG.%)	MCHOL. (MG.%)
4	6,000	100	176	380	270	9.20	15.60	2.54
5	6,270	72	147	280	300	11.40	9.90	3.30
7	8,400	102	207	300	357	13.05	9.30	3.03
8	4,800	112	144	330	210	10.20	10.80	2.50
9	4,200	64	102	260	242	7.70	6.20	1.98
10	5,000	70	118	200	266	8.50	6.60	2.44
Average	5,778	86.7	149	291.7	274	10.01	9.73	2.63
Isolated tissue								
6	3,500	80	160	147	240	6.40	5.00	1.42
7	3,600	70	122	145	254	8.40	6.00	1.50

For key to abbreviations see text.
*Phosphotungstic acid precipitate.

Isolated lymphoma tissue presented a somewhat different composition from lymphoma spleen. Total proteins were not increased, but were relatively

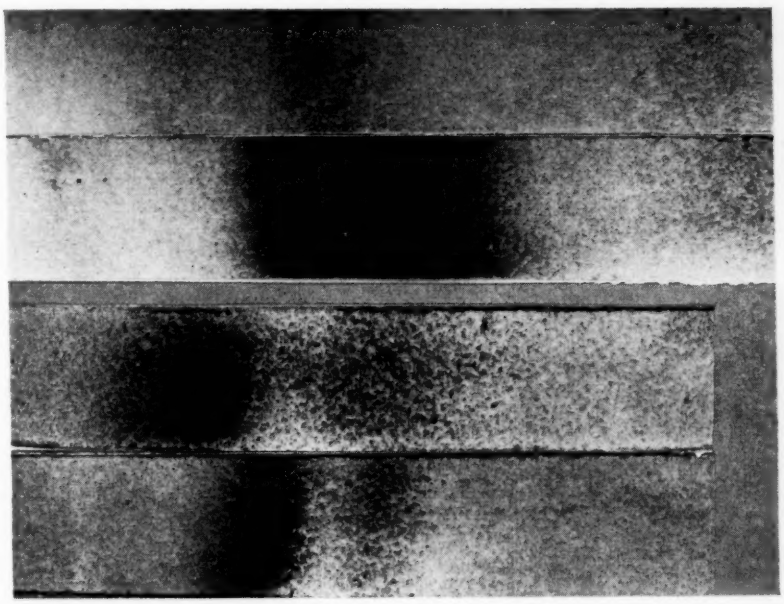

Fig. 1.—Electrophoretic pattern of soluble protein from normal splenic tissue and lymphoma tissue. *A*, Protein stain; *B*, glycoprotein stain.

In each group the upper strip represents normal splenic tissue, the lower strip lymphoma tissue. Note: (1) normal splenic tissue contains 2 peaks in γ and β position, indicated by both protein and glycoprotein stain. No definite albumin component is present. (2) Splenic tissue from patients with lymphoma is much richer in stainable material. Major peaks are present in α_2, β_1, and β_2 position; a small peak is found by protein stain in the α_1 albumin position.

rich in hexoses and hexosamine. Phosphotungstic acid precipitable protein, however, was greatly increased as compared to total protein; also, it was relatively poor in hexoses and hexosamine.

Electrophoretic studies of suspensions of homogenized lymphoma splenic tissue showed that, as with normal splenic tissue, there was preferential migration of proteins in the alpha-2 and beta zones. A new component, however,

was found in position alpha-1, the significance of which is under investigation at present. Similar pattern was observed in separated Hodgkin's tissue (Fig. 1).

Leukemia Spleens (Table IV).—Results were very similar to those observed in lymphoma tissue. The only significant difference was a lower cholesterol content in the protein fraction.

TABLE IV. PROTEINS AND PHOSPHOTUNGSTIC ACID PRECIPITATE ("MUCOPROTEIN FRACTION") IN LEUKEMIA: COMPARISON OF BLOOD AND SPLENIC TISSUE FINDINGS

CASE NO.	DIAGNOSIS		PROTEIN FRACTION T.P. (MG.%)	T.PH. (MG.%)	T.HA. (MG.%)	T.CHOL. (MG.%)	"MUCOPROTEIN FRACTION"* MP. (MG.%)	MH. (MG.%)	MHA. (MG.%)	MCHOL. (MG.%)
11	Histiocytic leukemia	Blood	7,200	147	140	200	310	42.50	12.75	1.30
		Spleen	7,050	138	234	146	420	6.8	13.60	1.20
12	Erythroblastic leukemia	Blood	7,000	160	140	160	123	20.91	18.70	2.00
		Spleen	7,800	126	156	150	306	11.40	14.73	1.80
13	Chronic malignant reticuloendotheliosis	Blood	7,700	145	130	180	130	34.30	14.50	3.80
		Spleen	5,550	60	216	170	295	11.40	11.85	2.25

For key to abbreviations see text.
*Phosphotungstic acid precipitate.

TABLE V. PROTEINS AND PHOSPHOTUNGSTIC ACID PRECIPITATE ("MUCOPROTEIN FRACTION") IN SPLEENS OF PATIENTS WITH MISCELLANEOUS DISEASES

CASE NO.	DIAGNOSIS	PROTEIN FRACTION T.P. (MG.%)	T.PH. (MG.%)	T.HA. (MG.%)	T.CHOL. (MG.%)	"MUCOPROTEIN FRACTION"* MP. (MG.%)	MH. (MG.%)	MHA. (MG.%)	MCHOL. (MG.%)
14	Acquired autoimmune hemolytic anemia	3,000	70	120	120	200	8.4	14.8	1.10
15	Fibrous congestive splenomegaly	8,250	63	186	206	270	12.45	22.8	3.75
16	Brucellosis; hemolytic anemia	6,900	117	195	185	339	13.35	22.8	3.45
17	Scleroderma thrombocytopenic purpura	8,700	90	249	207	345	10.5	12.3	3.30
18	Sarcoidosis; thrombocytopenic purpura	6,000	75	159	200	366	12.75	13.4	2.34
19	Aplastic anemia; hyperhemolysis	7,500	90	180	190	330	13.2	14.76	2.10
20	Congenital spherocytosis	4,830	90	135	210	300	6.4	9.8	3.48
21	Hemoglobinopathy	3,800	90	166	175	224	7.0	13.6	1.30
22	Myeloid metaplasia; rupture of spleen	6,150	111	180	220	357	7.6	11.2	3.03
	Average	6,125.5	88.4	174.4	190	303.4	10.18	15.05	2.54

For key to abbreviations see text.
*Phosphotungstic acid precipitate.

Spleens From Patients With Miscellaneous Diseases (Table V).—This group served as a control group to assess the specificity of the findings in

lymphoma and leukemia. It included a number of conditions where splenectomy was performed for the relief of excessive hemolysis or of thrombocytopenia, or because of traumatic rupture of the organ. Independently of the pathology involved, splenic tissue was rich in total proteins and phosphotungstic acid precipitable fraction. Unlike lymphoma and leukemia, however, values of hexoses, hexosamine and cholesterol followed closely those of the two main fractions.

A Comparison of Values of Serum and Splenic Tissue in Lymphoma (Table VI).—A group of 4 patients were selected where splenic tissue and serum had been simultaneously obtained under optimal conditions (surgical specimens). Total proteins and hexosamine were slightly higher in serum than in tissue. Total protein-bound hexoses were approximately twice as high in serum as compared to splenic tissue; the reverse was true of protein-bound cholesterol. The phosphotungstic acid precipitable fraction was comparatively more abundant in splenic tissue than in serum; this tissue fraction, however, was poor in hexoses and hexosamine and very poor in cholesterol as compared to blood.

TABLE VI. PROTEINS AND PHOSPHOTUNGSTIC ACID PRECIPITATE ("MUCOPROTEIN FRACTION") IN LYMPHOMA: COMPARISON OF SERUM AND SPLENIC TISSUE FINDINGS*

CASE NO.		PROTEIN FRACTION				"MUCOPROTEIN FRACTION"†			
		T.P. (MG.%)	T.PH. (MG.%)	T.HA. (MG.%)	T.CHOL. (MG.%)	MP. (MG.%)	MH. (MG.%)	MHA. (MG.%)	MCHOL. (MG.%)
4	Blood	6,200	158	95	150	158	10.8	18	4.5
	Spleen	6,000	176	100	380	270	9.2	15.6	2.54
5	Blood	6,000	200	165	160	200	17.0	16	9.5
	Spleen	6,270	72	147	280	300	11.4	9.9	3.30
9	Blood	7,300	221	155	145	190	15.7	9.3	5.9
	Spleen	4,200	64	102	260	242	7.7	6.2	1.98
10	Blood	7,700	200	175	180	204	7.7	13.6	7.9
	Spleen	5,000	70	118	200	266	8.5	6.6	2.44
Normal	Serum	7,340	109.5	98.6	200	98.8	6.5	11.3	2.9
	Spleen	3,585	55	105.4	143.7	113.1	7.47	10.55	1.76
Lymphoma	Serum	6,800	197.2	147.5	159	188	12.8	14.22	6.9
	Spleen	5,367	95.5	114.2	292	269.5	9.2	9.52	2.56
Serum	Normal	7,340	109.5	98.6	200	98.8	6.5	11.3	2.9
	Lymphoma	6,800	197.2	147.5	159	188.0	12.8	14.22	6.9
Spleen	Normal	3,585	55.0	105.4	143.7	113.1	7.47	10.55	1.76
	Lymphoma	5,367	95.5	114.2	292	269.5	9.20	9.52	2.56

For key to abbreviations see text.

*Normal values are entered for purpose of comparison. The second part of the table shows comparative values of serum and splenic tissue in normal individuals and in patients with lymphoma. For splenic tissue, normal values are those of Table II; serum values are those recorded in other publications.[1, 3, 9]

†Phosphotungstic acid precipitate.

DISCUSSION

The interpretation of the findings presented in this article should be entertained with great caution. From a theoretical viewpoint, our conclusions were made difficult by the uncertainty of nomenclature and the chemical nature

of mucoproteins. The phosphotungstic acid precipitate of tissue, even more than of serum, was likely to yield a fraction where the main constituents, mucoproteins, were mixed with a number of other tissue components. Moreover, techniques used involved repeated washing and manipulation of cells; thus, homogenates might have contained only more resistant cells; their number, in turn, may have changed greatly in number from normal to diseased state and in various conditions. Also, solubility and degree of recovery of proteins and other tissue constituents might have varied greatly because of noncontrollable influences. At their best, therefore, our results have mostly the value of comparing results in normal and in certain pathologic states.

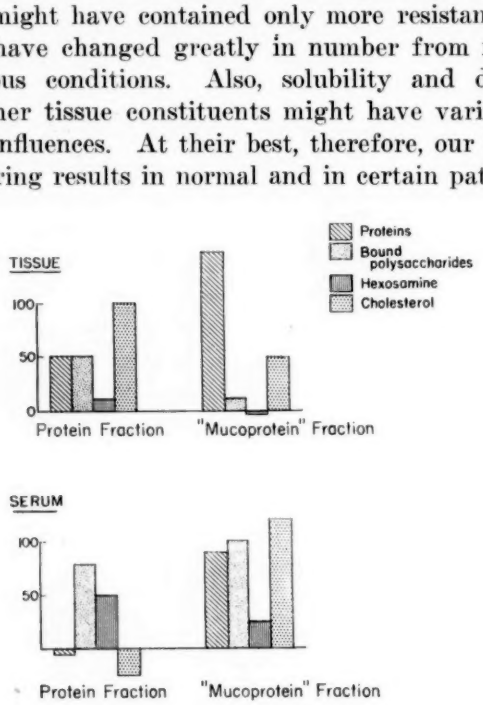

Fig. 2.—A comparison of various constituents of protein and phosphotungstic acid precipitate ("mucoprotein fraction") in serum and splenic tissue of patients with lymphoma. Note: (1) Tissue fraction is rich in cholesterol, poor in hexosamine; serum protein fraction is rich in hexoses and hexosamine, poor in cholesterol; (2) "mucoprotein fraction" is abundant in both lymphoma serum and spleen; tissue "mucoprotein fraction" is poor in hexoses, hexosamine, cholesterol; serum "mucoprotein fraction" is poor in hexosamine, rich in cholesterol. Findings suggest in lymphoma: (1) abnormal distribution of various substances between serum and tissue; (2) possibly, abnormal composition of proteins and other tissue and serum constituents.

With these limitations in mind, a few comments are warranted. Total proteins, total nonglucosamine protein-bound hexoses and total cholesterol* were elevated in all diseases affecting the spleen, probably because of increased cellularity of the organ. Hexosamine, however, was elevated only slightly. Even more striking was the high content of phosphotungstic acid precipitable fraction in diseased splenic tissue. In lymphoma and leukemia, however, there were important additional qualitative changes, since this fraction was relatively poor in hexoses and hexosamine and, less strikingly, in cholesterol. Thus, quantitative and qualitative changes in the composition of the total protein fraction and of the "mucoprotein fraction" in pathologic splenic tissue as compared to normal tissue suggested significant changes in their composition, possibly specific for different disease states.

*We have previously shown that accumulation of cholesterol in lymphoma tissue may be related to parallel increased content of cholesterol-carrying beta globulins.[3]

The comparison of tissue and serum values was also of some interest, although, perhaps, open to many objections. Our results showed a relatively fixed proportional distribution of total protein, phosphotungstic acid precipitate, and constituents of both fractions between tissue and serum from normal individuals or from patients with miscellaneous diseases. In lymphoma, however, there was a striking dissociation of findings between splenic tissue and serum (Fig. 2). Total protein fraction of tissue was rich in cholesterol, poor in hexosamine; total protein fraction of serum, on the other hand, was rich in hexoses and hexosamine but poor in cholesterol. The phosphotungstic acid precipitate from serum was rich in hexoses and cholesterol. The tissue precipitate was poor in hexoses and cholesterol. The mechanism of the apparently altered equilibrium of certain serum and tissue constituents in lymphoma is being investigated at present.

SUMMARY

1. This article reports a study of some constituents of the total protein and phosphotungstic acid precipitable fraction ("mucoprotein fraction") of serum and splenic tissue in normal individuals and patients with lymphoma, leukemia, and miscellaneous disorders.

2. In all pathologic cases studied splenic tissue was rich in total protein. The protein fraction of lymphoma tissue presented the strikingly distinctive feature of a very high cholesterol content. Total hexosamine was relatively low in splenic tissue of lymphoma and leukemia.

3. Pathologic splenic tissue was also rich in phosphotungstic acid precipitable material ("mucoprotein fraction"). When obtained from lymphoma and leukemic splenic tissue this fraction appeared relatively poor in hexoses, hexosamine, and cholesterol.

4. Comparison of splenic tissue and blood values showed a series of significant findings in lymphoma. Cholesterol of total proteins was high in tissue, low in blood. Also, total protein-bound hexoses were much higher in serum than in splenic tissue; phosphotungstic acid precipitate from tissue contained relatively less hexoses, hexosamine, and cholesterol than the corresponding fraction from serum.

5. Results suggested (a) quantitative and qualitative changes of total protein and "mucoprotein fraction" of serum and tissue in disease; (b) possibly, abnormal composition of these fractions in disease, particularly lymphoma.

We wish to acknowledge the assistance of the Departments of Medicine, Pathology, and Surgery of the Massachusetts General Hospital, Boston, for making Patients 15 and 16 available for study.

Dr. James H. Graham, Saint Elizabeth's Hospital, and Dr. Benjamin Castleman, Massachusetts General Hospital, kindly interpreted the pathology of the organs involved in this study.

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STUDIES ON PLATELETS XIX: CAROTENOID PIGMENTS IN HUMAN PLATELETS

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IN THE course of unrelated experiments, it was observed by one of us that buttons of human platelets obtained from a large number of donors and stored at 4° or at –20° C. would often assume a yellowish-brown color. This observation suggested the presence of pigment precursors or of bound pigments in human platelets. Further study indicated that an ethyl ether extract of these platelets[1] which had been stored for one month at –20° C. contained yellow pigments and an ultraviolet absorbing material which was spectrally similar to vitamin A and in addition gave a positive Carr-Price reaction. Attempts to reidentify vitamin A in intact platelets or in their extracts has since failed. Repetition of the work, however, has consistently revealed the presence of carotenoid pigments in extracts of platelets.

MATERIALS AND METHODS

Preparation of Platelet-Rich Plasma and of Suspensions of Isolated Platelets.—Blood was collected from healthy or polycythemic individuals in plastic bags containing ACD solution.† In several other experiments, blood was collected directly in silicone-coated bottles containing 1/10 volume of 0.2 M sodium citrate. Platelets and plasma from 10 different donors were used in these studies. Platelet-rich plasma and triply saline washed platelet buttons were obtained as described.[2] Platelets were also obtained from rabbit blood collected in silicone-coated syringes containing sodium citrate by direct arterial or heart puncture. Techniques of preparation of rabbit platelets have been described earlier.[3]

Fractionation of Platelets and Pigment Extraction.—The platelets from 1 pint of blood were suspended in 2.5 ml. saline. To this, 7.5 ml. of ethyl ether containing 3.5 per cent ethanol was added. The mixture was shaken vigorously for 5 minutes, and then centrifuged at 3,000 r.p.m. for 30 minutes in cork-stoppered test tubes. Three layers were formed, as described earlier.[1] After discarding the top layer, the remaining solution was stored at –20° C. for 24 hours. This material was thawed, centrifuged at 3,000 r.p.m. for 30 minutes, and 3 new layers formed. The top layer appeared yellow after this treatment, and was saved for further analysis. An alternate technique consisted in adding 3 volumes of anhydrous sodium sulfate to a packed platelet button, and grinding until dry. The resulting powder was extracted with petroleum ether, and then with acetone to remove protein-bound pigments.

Analysis of Pigments.—The platelet extracts were transferred to petroleum ether, and chromatographed on alumina columns. The pigments which separated were identified both

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Supported in part by grant H-2132 from the National Institutes of Health.

Received for publication Jan. 2, 1957.

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by their adsorptive behavior and spectral characteristics. The concentration of the xanthophyll fraction (lutein) was obtained using the E (1%, 1 cm.)* value of 2,520 at 443 mμ. For the carotene fraction (a mixture of β-carotene and lycopene), E (1%, 1 cm.) of 2,115 at 440 mμ, the crossing point of two compounds, was used.

RESULTS

Direct spectrophotometric readings of the petroleum ether extract of human platelets revealed the presence of two absorption peaks at 445 and 470 mμ (Fig. 1). Similarly shaped curves were obtained for both the acetone extract, and the ethyl ether extract, when these had been transferred into petroleum ether.

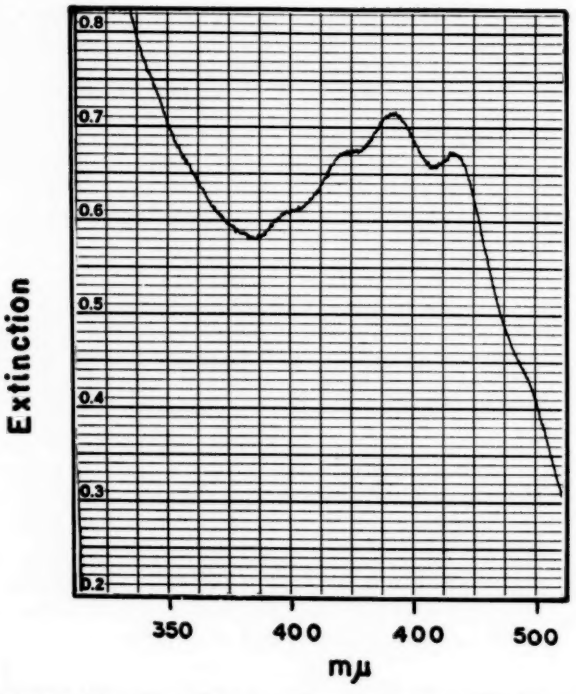

Fig. 1.—Spectrophotometric recording of a petroleum ether extract of human platelets. The extinction (ordinate) is plotted directly against wave length (abscissa).

Alumina chromatography at room temperature resolved these extracts into two colored fractions. The first, eluted with a solution of petroleum ether containing 4 per cent acetone, had an absorption spectrum indicative of a mixture of β-carotene and lycopene (Fig. 2, *a*).[4] When this fraction was saponified and then rechromatographed, it again was eluted with the 4 per cent acetone solution, indicating that there were no xanthophyll esters present. Following the elution of the first fraction, an additional yellow zone could be removed by the use of a petroleum ether solution containing 40 per cent acetone. This fraction, both by its adsorptive behavior and spectrum (Fig. 2, *b*), was identified as lutein.

*The extinction (log I_0/I) of a solution containing 1 Gm. solute/100 ml. solution when read in a 1 cm. light path.

None of these extracts showed a positive Carr-Price reaction, possibly because of the low concentration of the materials analyzed. Since, however, vitamin A had been found in a single previous preparation, the problem arose as to whether vitamin A could be formed from the carotenoids present in the platelets. Long term storage at –20° C., or incubation for 2 hours at temperatures up to 37° C. of either platelets or their extracts failed to be followed by the appearance of vitamin A.

Inasmuch as the plasma lipoproteins carry carotenoids,[4] it was felt that the carotenoids found in platelets might be due to lipoprotein contamination. Although this possibility cannot be eliminated rigorously, it appeared unlikely, since the amount of pigment recovered from the platelet extract was quite consistently proportional to the number of platelets used, irrespective of the

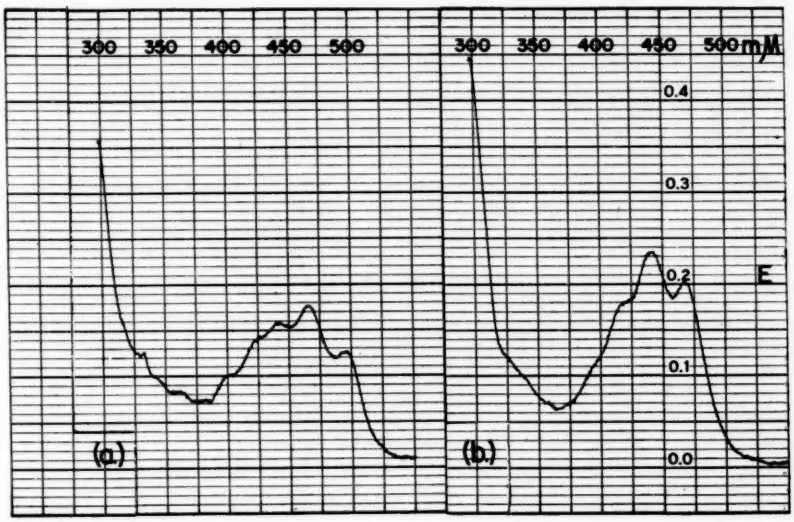

Fig. 2.—Spectrophometric recording of a petroleum ether extract of human platelets after chromatographic separation on an alumina column. *a*, β-carotene-lycopene mixture eluted with 4 per cent acetone in petroleum ether. *b*, Protein eluted with 40 per cent acetone in petroleum ether.

presence of alimentary hyperlipemia. Moreover, in an additional experiment, platelets were obtained from the same donor before and after clarification of plasma from fats by the intravenous administration of 50 mg. of heparin. The amount of pigments finally obtained was very similar in both instances.

Treatment of rabbit platelets as described indicated the presence of a pigment with λ max. at 398 and 420 mμ. The nature of this pigment is being investigated at present.

DISCUSSION

The findings presented in this article indicate that carotenoid pigments (xanthophyll and a β-carotene-lycopene mixture) are found in human platelets under certain experimental conditions. These pigments are among those also found in plasma. It was, therefore, important to compare the concentration of these carotenoids in platelets to the concentration in plasma. Platelets from

one pint of blood contained 2.9 μg of xanthophyll and 2.5 μg of β-carotene-lycopene mixture. By comparable techniques[4] plasma from one pint of blood contained the same pigments in amounts of 94 and 170 μg, respectively. Since platelets usually represent 1 per cent by volume of plasma, a slightly higher concentration of these pigments in platelets than in plasma was apparent. Thus, platelets may concentrate carotenoids, contributing to their transport in the blood stream. This function of transport, thus far, has been considered limited to lipoproteins and chylomicrons.

Unfortunately, however, the relationship of carotenoid pigments to platelets could not be clarified further. It appeared unlikely that the presence of carotenoids in platelets might have been due to free diffusion from plasma, because the pigments exhibited relatively higher concentration in thrombocytes as compared to plasma. Also, multiple washings with water and even a single extraction with ether did not affect the concentration of pigments in platelets. It was possible, on the other hand, that platelets, whose structure could be visualized as a giant lipoprotein complex, might have adsorbed circulating carotenoid pigments. The resulting bondage was apparently a strong one, to be dissolved only by the complete destruction of the platelet body. A similar situation has been described for other constituents of platelets, like antifibrinolysin[5] and 5-hydroxytryptamine creatinine sulfate.[6]

Finally, the status of carotenoid pigments found in platelets was not clearly understood. Storage and strong chemical "treatment" were needed for their demonstration. Whether these procedures liberated or, perhaps, produced these pigments from other substrates; whether carotenoids were present in normal human platelets as such or, rather, as precursor substances remained undetermined.

SUMMARY

1. Human platelets have been found to contain lutein, lycopene and β-carotene. There is moderate concentration of the pigments in the platelet body as compared to plasma.

2. The finding is best explained by the known ability of platelets to absorb many constituents of the circulating plasma. Thus, platelets may contribute to the transport of carotenoid pigments in the blood stream.

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COAGULATION DEFECTS IN LEUKEMIA

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IT HAS been over thirty years since Minot and Buckman[1] published their classic article on bleeding in leukemia. Yet, in spite of a great deal of investigation, little has been learned of the etiology of the hemorrhagic diathesis in the various leukemias and other myeloproliferative disorders. Of course, in the presence of marked thrombocytopenia the cause is obvious and points the way toward the proper treatment. However, it is an uneasy situation for the clinician when his patient develops some hemorrhagic manifestation with a normal or even elevated platelet count. The etiology of the bleeding in this type of leukemic patient has been obscure and many explanations have been advanced. One of the earliest was decreased resistance of the vascular wall due to anoxemia and leukemic infiltration.[1] Although this may be a factor at times, it is not a common cause for the bleeding seen in the nonthrombopenic patient with leukemia.[2]

The possibility that the bleeding is due to qualitative platelet defects has been advanced without very firm evidence.[3] Why one patient bleeds while another, with comparable thrombocytopenia, does not is puzzling. Platelet and plasma serotonin determinations have not been of aid in this problem.[4, 5] Hypoprothrombinemia and AC globulin deficiency due to liver damage have been mentioned.[6] Again, these must be uncommon since most patients with chronic leukemia do not have abnormal liver function tests and have normal prothrombin times.[7] Fibrinogenopenia and fibrinolysins have been demonstrated in some cases,[8, 9] but this must be rare since Pisciotta and Schulz[10] failed to find one such instance in 40 patients with leukemia. Similarly, circulating anticoagulants are responsible in an occasional case only.[3] Finally, the presence of a serum polysaccharide has been postulated to account for the onset of bleeding in thrombocytopenic patients with leukemia.[11] However, fever and infection are not commonly associated with hemorrhagic manifestations in these patients.[3]

With all these theories, it is obvious that the cause for hemorrhage in leukemia is often obscure.[12, 13] As a result, the clinician is frequently at a loss as to appropriate therapy.

This study was undertaken in an attempt to elucidate the coagulation defect in leukemia and other myeloproliferative disorders.

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Supported in part by Parke, Davis & Co., U. S. Public Health Service, and The Gladys F. Bowyer Fund.

Received for publication Jan. 4, 1957.

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TABLE I

DISEASE	NO. OF PATIENTS	NO. OF PATIENTS WITH BLEEDING	AVERAGE PLATELET COUNT	AVERAGE BLEEDING TIME	AVERAGE CLOTTING TIME		NO. WITH ABNORMAL CLOT RETRACTION	AVERAGE PRO-THROMBIN TIME	AVERAGE PRO-THROMBIN CON-SUMPTION	NO. WITH AB-NORMAL TGT	PLATELET DEFECTS IN TGT	
					GLASS	SILICONE						
NORMAL	25		330,000	2′-3′	8′-15′	13′ (6′-19′)		13.5″	34.2″			
Leukemoid	3	0	421,000	2′40″		18′	1	16″	45.5″	0	Qual.	2
Polycythemia rubra vera	4	2	568,000	1′45″		23′30″	0	14.7″	31.5″	1	Qual.	2
Acute myelocytic leukemia	1	1	3,100	10′		20′	1	19.5″	12.5″	1	Quan.	1
Acute lymphocytic leukemia	1	0	45,000	3′ 6″	13′	40′+	1	16.5″	18.5″	1	Qual.	1
Monocytic leukemia	2	1	115,000	4.5′		11′40″	2	14.5″	41.8″	2	Qual.	1
											Qual. and Quan.	1
Monomyelocytic (Naegeli) leukemia	1	1	3,000	10′+	40′+	40′+	0	17.5″	10″	1	Quan.	1
Chronic myelocytic leukemia	8	7	484,000	7′38″	10.1′	21′12″	5	14″	28.2″	6	Qual.	3
											Quan.	1
											Qual. and Quan.	2
Chronic lymphocytic leukemia	19	6	154,000	2′29″	7′	21′23″	6	13.7″	28.6″	12	Qual.	12

J. Lab. & Clin. Med. August, 1957

METHODS

Nineteen unselected patients with chronic lymphocytic leukemia, 8 with chronic myelocytic leukemia, one with acute lymphocytic, one with acute myelocytic, one with monomyelocytic (Naegeli) type, and 2 with monocytic leukemias were studied with a battery of clotting tests. Four patients with polycythemia rubra vera and 4 with leukemoid reactions and myeloid metaplasia were also investigated.

In addition, the same coagulation studies were performed on a group of 25 normal controls. These included members of the staff and patients with no apparent hematologic disorder, liver disease, congestive failure, or other disease which might disturb the clotting mechanism. One and frequently two normal controls were also done concurrently on days that patients with the myeloproliferative disorders were studied.

Only siliconized needles and syringes were used. Venipunctures were initiated with one syringe, which was then discarded, and only the blood obtained with a second syringe was used for the clotting studies.

Platelet counts were performed by mixing equal quantities of venous blood (0.5 ml.) and Rees-Ecker solution[14] without dye. Smears were then prepared as for ordinary blood smears and, after drying, were stained with Wright's solution. A modified Miller ocular disk reticulocyte technique[15] adapted for platelets was used in counting. This method is excellent in that the results are resproducible with accuracy. They may be rechecked at will since the smears are filed for further study.

Prothrombin times were performed by use of human brain thromboplastin and resulted in a clotting time of 13.5 seconds normally. Tests for accelerator globulin[3] and serum prothrombin conversion accelerator[3] were done when indicated.

The prothrombin consumption test used was a modification of the Owren method.[16] Two milliliters of blood were delivered into a standard tube which was inverted 10 times and then placed in a 37° C. water bath for one hour after clotting. The serum was then separated by centrifugation and a one-stage prothrombin time done.

Thromboplastin generation tests (TGT) were performed according to Biggs and Douglas,[17] with some modifications similar to those recently suggested.[18] Incubation was not carried beyond 6 minutes. The platelet suspension as prepared for the thromboplastin generation test in this study contained per cubic millimeter between 3 and 4 times the number of platelets in the patient's blood. They were washed twice with saline and resuspended in a volume of saline equal to one-third of the original platelet-rich plasma suspension. The platelet counts given in this paper were those found in whole blood. The effect of the patient's platelets on thromboplastin generation was measured by using them in a system containing normal serum, barium sulfate-treated normal plasma, and platelet-poor plasma substrate as well as in a system containing only the patient's reagents. The platelet suspension was also diluted or concentrated to correspond to an appropriate whole blood level and was used in both systems.

The initial tests in every patient were performed by use of all of the patient's reagents both in his own substrate and in normal substrate plasma. If there was no appreciable difference, the normal substrate was used for all subsequent tests in that particular patient. Both substrate plasmas were used to aid in the detection of circulating anticoagulants of the antithromboplastin type. In addition, tests for abnormal anticoagulant activity were performed, employing varying proportions of normal and patient's plasma.[19]

Tubes containing 2 ml. of blood were examined by visual inspection for clot retraction after one hour at 37° C. and for fibrinolytic activity after 24 hours. One milliliter aliquots of blood were delivered into glass and siliconized 1 × 10 cm. test tubes for the whole blood clotting times by the Lee-White method.[13] These were done at room temperature.

RESULTS

Table I summarizes the results obtained in the 39 patients studied. It is to be noted that the average platelet count in the patients with chronic

TABLE II

DISEASE	PATIENT			AGE	HISTORY OF BLEEDING	PLATELET COUNT	BLEEDING TIME	CLOTTING TIME		CLOT RETRACTION	PROTHROMBIN TIME	PROTHROMBIN CONSUMPTION	THROMBOPLASTIN GENERATION	PLATELET DEFECT IN TGT
								GLASS	SILICONE					
Chronic lymphocytic leukemia	B.	♂	W	44	−	107,000	1′45″		30′	Poor	15″	14″	Abnormal	Qual.
	Ba.	♂	W	90	−	155,000	1′47″		18′	Poor	14″	17″	Abnormal	Qual.
	B., L.	♂	W	62	−	172,000	4′13″		22′	Good	15″	21″	Normal	None
	Bo.	♂	W	54	−	74,000	1′30″		19′	Poor	15″	13.5″	Normal	None
	C.	♂	W	64	+	97,000	1′13″		40′+	Poor	10.5″	25″	Abnormal	Qual.
	D.	♂	W	61	−	164,000	2′		8′	Good	15″	34″	Normal	None
	Du.	♂	N	49	+	161,000	3′		21′22″		12.5″	13.5″	Abnormal	Qual.
	F.	♂	W	64	+	183,000	2′		26′	Poor	13.5″	43.5″	Abnormal	Qual.
	Go.	♂	W	94	−	79,000	2′		18′	Poor	15″	36″	Abnormal	Qual.
	G.	♂	N	61	−	163,000	1′30″	7′	16′	Good	16″	81″	Normal	None
	Ha.	♀	W	76	−	263,000			22′		13.5″	36″	Normal	None
	H.	♂	N	38	+	140,000	2′ 5″		25′		12″	13″	Abnormal	Qual.
	Hu.	♂	W	60	+	81,000	3′15″		24′		14.5″	23″	Abnormal	Qual.
	J.	♂	W	68	−	297,000	1′30″		22′17″	Good	11.5″	26″	Normal	None
	J., S.	♂	N	62	−	140,000	2′40″		22′56″		14.5″	26″	Abnormal	Qual.
	M.	♂	W	60	−	329,000	5′22″		14′		12.5″	28″	Normal	None
	P.	♂	W	60	−	63,000	3′30″				13.5′	22.5″	Abnormal	Qual.
	S.	♂	N	76	−	155,000				Good	14″	33.5″	Abnormal	Qual.
	W.	♂	W	51	+	104,000	3′		11′	Good	13″	37″	Abnormal	Qual.
Chronic myelocytic leukemia	C.	♂	W	78	+	12,000	6′	12′	30′+	Poor	13.5″	14″	Abnormal	Quan.
	J.	♀	W	70	+	1,365,000		9′	6′30″	Good	14.5″	19″	Abnormal	Qual.
	K.	♂	W	75	+	7,000	10′+	9′30″	24′	Poor	14″	12.5″	Abnormal	Qual.-Quan.
	M.	♂	W	52	+	11,000	10′30″		16′30″	Poor	16″	14″	Abnormal	Qual.-Quan.
	P.	♂	W	63	+	1,825,000	4′50″		27′	Good	15.5″	30″	Abnormal	Qual.
	Pi.	♂	W	34	+	30,000	12′		21′	Poor	12″	13.5″	Abnormal	Qual.
	S.	♂	W	72	−	538,000	2′30″		24′	Poor	14″	33″	Normal	None
	W.	♂	W	34	+	182,000					12.5″	89.5″	Normal	None
Polycythemia rubra vera	E.	♂	W	49	+	451,000	2′30″		32′	Good	17″	32.5″	Abnormal	Qual.
	G.	♂	W	67	+	1,197,000			32′	Good	14″	19″	Normal	Qual.
	H.	♂	W	50	−	221,000	1′		18′	Good	14.5″	41″	Normal	None
	W.	♂	W	69	−	322,000			12′	Good	13.3″	13″	Normal	None
Leukemoid	A.	♂	W	65	−	428,000	1′		20′	Good	17″	65″	Normal	Qual.
	M.	♂	W	65	−	611,000	5′		14′	Good	15″	20.5″	Normal	Qual.
	T.	♂	W	71	−	223,000	2′		20′	Good	15″	51.5″	Normal	None

lymphocytic leukemia was reduced, whereas it was somewhat elevated in the small number of patients with chronic myelocytic leukemia. In spite of this, the average bleeding time seems prolonged in the latter. However, from Table II it is apparent that the prolonged bleeding times occurred in patients with thrombocytopenia. The prothrombin consumption time was short in both groups. Platelet defects, as measured in the TGT in chronic lymphocytic leukemia, when present, were qualitative in nature. Concentration of the platelets in those patients with quantitative reductions to what would correspond to normal whole blood levels did not result in normal thromboplastin generation.

Table II lists the individual patients studied and the results of their clotting tests. Fibrinolytic activity was not found in any case. A circulating anticoagulant was thought to be present in only one individual. The latter was a patient (M.) with chronic myelocytic leukemia with an associated platelet defect. Thromboplastin generation was poor both in the patient's plasma and in normal substrate plasma. Correction was not obtained with substitution of any normal or combination of normal reagents. However, the test for anticoagulant activity was equivocal. One patient (J.) with chronic myelocytic leukemia had slightly deficient thromboplastin generation which was corrected only when either normal serum or plasma was used along with normal platelets.

In general, prothrombin times were not markedly prolonged. There were no instances of AC globulin or serum prothrombin conversion accelerator deficiency.

Of the 39 patients investigated, 18 had a history of bleeding and, of these, 17 had at least one abnormal clotting test. The latter also included 16 with abnormal thromboplastin generation. Among the 21 patients with no history of bleeding, 8 had abnormal TGT. On the other hand, one-third of the patients with poor thromboplastin generation tests gave no history of bleeding.

TABLE III

DISEASE	ABN. TGT + BLEEDING	NORMAL TGT + NO BLEEDING	NORMAL TGT + BLEEDING	ABN. TGT + NO BLEEDING
Leukemoid		3		
Polycythemia rubra vera	1	2	1	
Acute myelocytic leukemia	1			
Acute lymphocytic leukemia				1
Monocytic leukemia	1			1
Monomyelocytic (Naegeli) leukemia	1			
Chronic myelocytic leukemia	6	1	1	
Chronic lymphocytic leukemia	6	7		6
Total	16	13	2	8

These results are listed in Table III. It is important to note that in the 24 patients with poor thromboplastin generation, 11 had normal prothrombin consumption. It is apparent that a normal prothrombin consumption time, especially in the presence of a prolonged prothrombin time, may be misleading and does not exclude an abnormality in thromboplastin generation.

Of the three patients with leukemoid blood pictures and myeloid metaplasia, none had a history of bleeding, although all had some abnormality in their clotting studies. One additional patient, not listed in the tables, had so-called "burned out" polycythemia rubra vera and a severe hemorrhagic diatheses. Thromboplastin generation was extremely poor. It was not possible, in this particular patient, to exclude the presence of myelocytic leukemia.

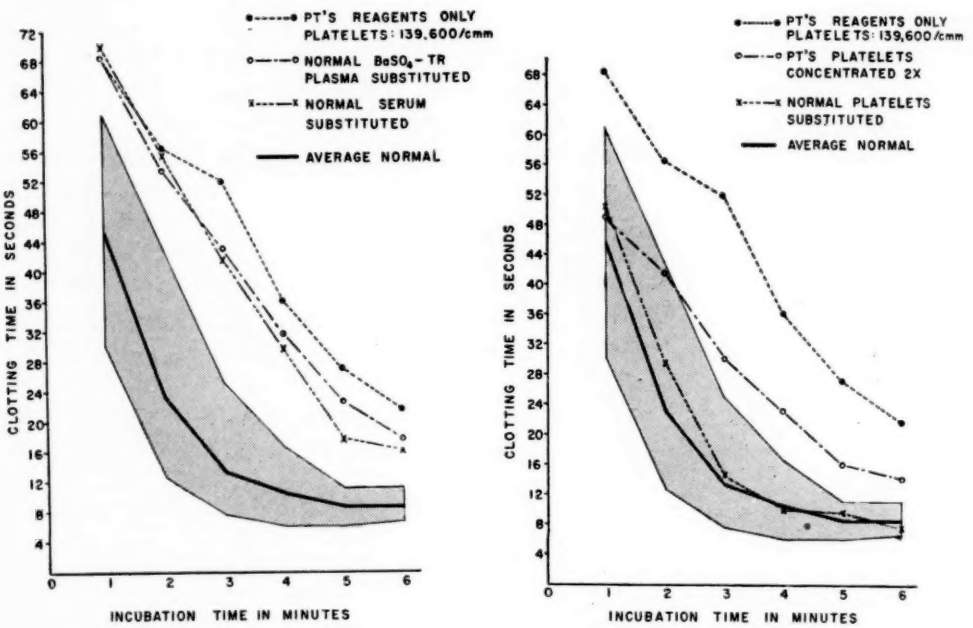

Fig. 1.—Patient (H.) with chronic lymphocytic leukemia and a qualitative platelet defect. Normal barium sulfate treated plasma and normal serum did not result in normal thromboplastin generation. Doubling of the patient's platelets improved the test only moderately. However, substitution of normal platelets resulted in normal thromboplastin generation. The shaded area indicates the range of thromboplastin generation in 25 normal controls.

As was to be expected, some of the platelet defects were found to be solely quantitative. However, qualitative defects as determined in the thromboplastin generation test were extremely common in these patients, particularly those with chronic lymphocytic leukemia. In this latter group, in fact, a quantitative reduction of platelets alone was in no case found to be responsible for an abnormal thromboplastin generation test.

Fig. 1 is an example of the type of platelet defect seen in chronic lymphocytic leukemia. Although the platelets were reduced in number, they were also qualitatively defective. Concentration to what would correspond to a normal value in whole blood did not result in a normal curve. Fig. 2 illustrates a similar defect in a patient with polycythemia rubra vera who had a slightly elevated platelet count.

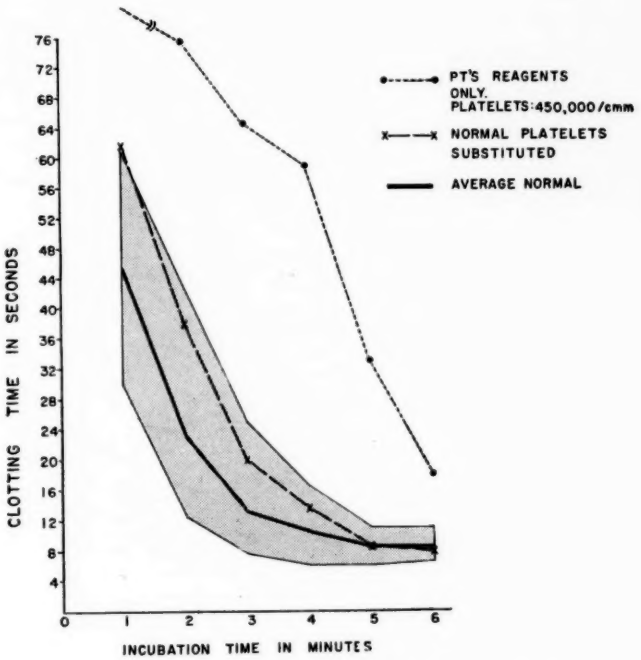

Fig. 2.—Polycythemia rubra vera. Poor thromboplastin generation in a patient (E.) with a history of bleeding and a slightly elevated platelet count (normal: 200,000 to 350,000). Normal thromboplastin generation was obtained when normal platelets were substituted.

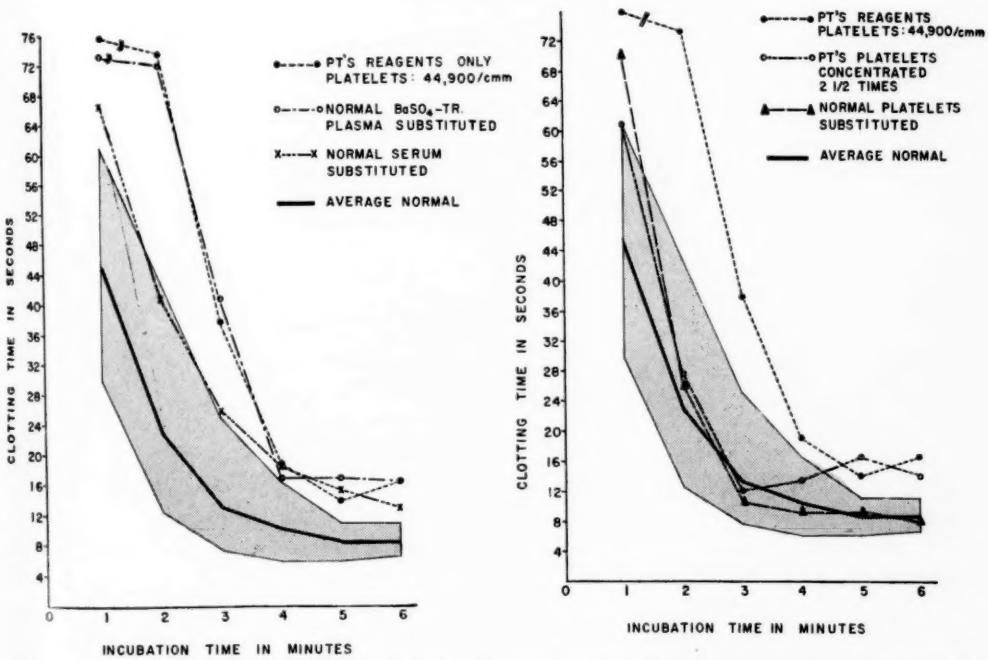

Fig. 3.—Acute lymphocytic leukemia. Improvement in thromboplastin generation as the patient's own platelets were concentrated to what would correspond to a platelet count in the blood of 125,000 per cubic millimeter. The platelet defect appears to be essentially a quantitative one.

Fig. 4.—Acute myelocytic leukemia (Patient B.). Thromboplastin generation in the presence of marked thrombocytopenia. Although the patient's own platelets were concentrated only to a level of 22,000 per cubic millimeter, thromboplastin generation was almost completely normal.

Fig. 5.—Abnormal thromboplastin generation in a patient (K.) with chronic myelocytic leukemia. Marked improvement occurred after concentration of the patient's platelets to a level of approximately 44,000 per cubic millimeter.

Fig. 3 illustrates the effect on thromboplastin generation of a quantitative platelet defect in a patient with acute lymphocytic leukemia. Concentration of the patient's platelets in the system yielded almost a normal curve. A similar situation in acute myelocytic leukemia is shown in Fig. 4. Although the platelet count after concentration corresponded to a whole blood count of approximately 22,000 per cubic millimeter, thromboplastin generation was nearly normal. Fig. 5 is an example of the same type of defect in chronic myelocytic leukemia.

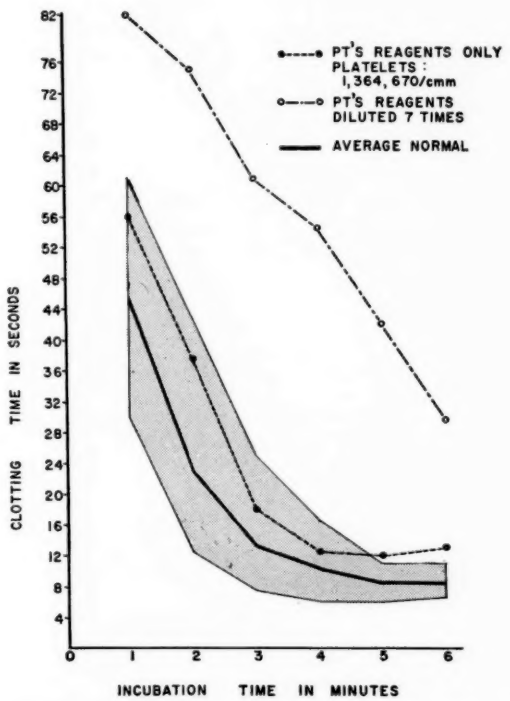

Fig. 6.—Chronic myelocytic leukemia (Patient J.). Demonstration of qualitative platelet defect only after the patient's platelets were diluted to a level of approximately 200,000 per cubic millimeter. Thromboplastin generation was normal in the presence of the excessive numbers of patelets but was poor at lower levels.

There were three patients with platelet counts above one million per cubic millimeter. One patient with chronic myelocytic leukemia had a qualitative platelet defect as demonstrated in Fig. 6. The excessive numbers of platelets permitted good thromboplastin generation. However, with dilution of these platelets to a normal level, thromboplastin generation became inadequate.

Another patient with excessive platelets had an abnormal TGT (Fig. 7) even without dilution. Fig. 8 is an example of a patient with chronic myelocytic leukemia and elevated platelets in which, even with dilution, thromboplastin generation was normal. Just the opposite situation is shown in Fig. 9 in a patient with myelofibrosis where dilution resulted in poor thromboplastin generation.

Fig. 5.—Abnormal thromboplastin generation in a patient (K.) with chronic myelocytic leukemia. Marked improvement occurred after concentration of the patient's platelets to a level of approximately 44,000 per cubic millimeter.

DISCUSSION

The results of this study are in agreement with previous impressions[1, 3, 6] that there is no constant relationship between the platelet count and the incidence of bleeding. A patient with a normal or elevated platelet count may bleed and a patient with thrombocytopenia may never have any hemorrhagic

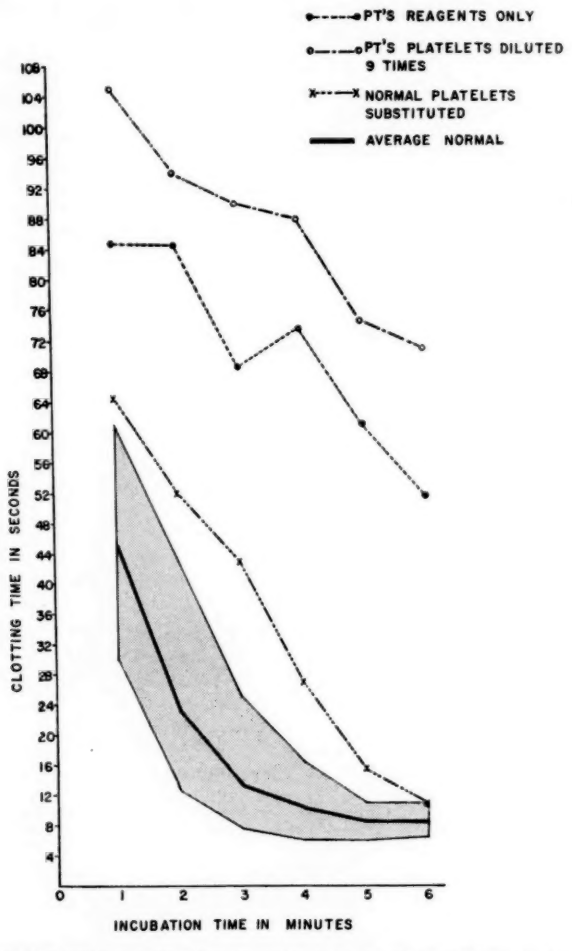

Fig. 7.—Abnormal thromboplastin generation in spite of a great increase in platelets (1,825,000 per cubic millimeter) in a patient (P.) with chronic myelocytic leukemia.

manifestations. Three patients with excessive platelets (over one million) had bleeding histories. On the other hand, one patient (P.) with chronic lymphocytic leukemia and a platelet count of 63,000 had never had any bleeding. Generally, however, a patient with thrombocytopenia is more likely to bleed.[20] Yet a patient with a normal or high platelet count often will demonstrate a hemorrhagic diathesis if these platelets are qualitatively defective. In fact, thrombocythemia is frequently associated with bleeding in polycythemia rubra vera, chronic granulocytic leukemia, and so-called hemorrhagic thrombocythemia.[5, 21, 22] Patients with elevated platelet counts may demonstrate

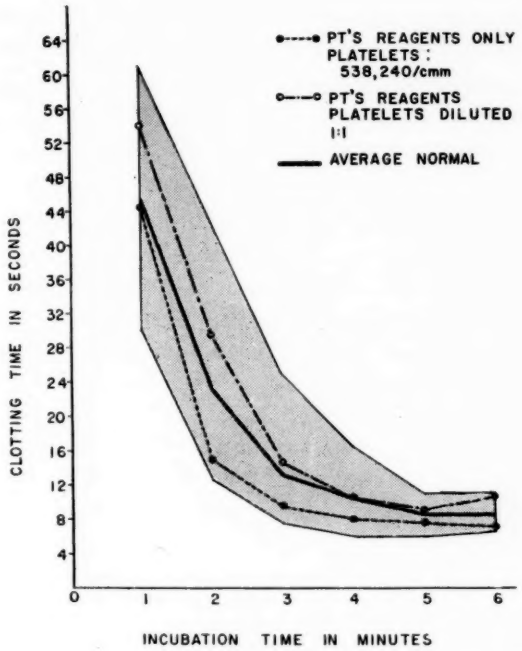

Fig. 8.—Chronic myelocytic leukemia (Patient S.). In spite of dilution of the platelets in this patient, thromboplastin generation did not become abnormal.

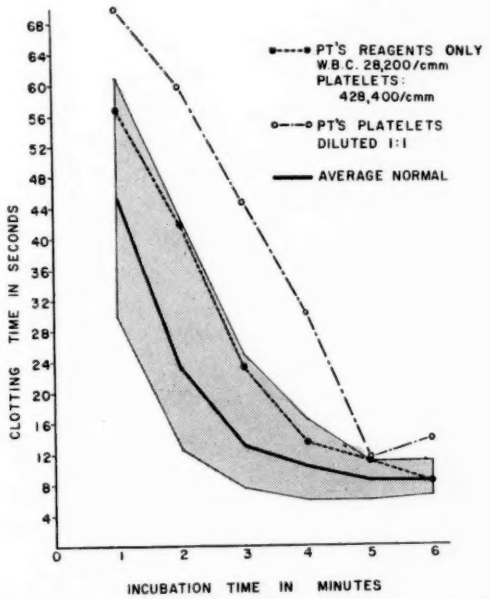

Fig. 9.—An example in a patient (A.) with myelofibrosis and a slightly elevated platelet count, where dilution of the platelets resulted in abnormal thromboplastin generation.

abnormal thromboplastin generation only after dilution of the platelets to more nearly normal levels, as shown in Fig. 5. Hardisty and Wolff[5] have recently reported similar observations in patients with hemorrhagic thrombocythemia. Finally, excess normal platelets may even possess anticoagulant activity in vitro.[21]

From the results obtained, it is not possible to determine at what platelet level bleeding will occur. The value of 60,000 to 75,000, usually mentioned,[1, 20] is of little importance by itself. Low numbers of platelets may be functionally quite adequate, while, as mentioned above, excessive platelets may be associated with hemorrhage. Evidence for this may be seen in the results of the thromboplastin generation tests and observations on clot retraction. Similar variation of the critical platelet level was reported in 1946 by Aggeler and his co-workers.[20] In this connection, in regard to platelets, it is important to point out the lack of correlation among the results obtained in the thromboplastin generation test, clot retraction (by visual observation), and the platelet count. To gauge platelet function, all these tests must be performed in addition to the bleeding time and tourniquet tests.

Although deficient platelet function, particularly as measured in the thromboplastin generation test, often seems to be associated with hemorrhage, it is only presumptive etiologic evidence and may not be solely responsible for the bleeding. Other factors, not measured in this study or hitherto unrecognized, may be even more important.

Although not statistically significant because of the small numbers of patients studied, it is interesting to note that only qualitative platelet deficiencies were found in the group of patients with chronic lymphocytic leukemia. Sixteen of the 19 had low platelet counts. Abnormal thromboplastin generation tests in these were not corrected when their platelet suspensions were concentrated. In the other groups of patients both qualitative and quantitative defects were present. In the small number of patients with chronic myelocytic leukemia, variation in the levels of platelets from case to case was especially marked. The characteristic decrease in chronic lymphocytic leukemia and the variation in chronic myelocytic leukemia have been noted before.[1] The explanations for this and for the qualitative changes in the platelets are not obvious. However, functional deficiencies, including those involving the hemostatic activities of platelets, should not be totally unexpected. Biochemical and physiological abnormalities of the other blood cells in leukemia have been reported previously.[23, 24]

SUMMARY

1. A group of 39 patients with various myeloproliferative disorders, including chronic myelocytic and lymphocytic leukemia, were studied with a battery of clotting tests.

2. Of 18 patients with a history of bleeding, 17 had one or more abnormal clotting tests, including poor thromboplastin generation in 16. Four had excessive platelets and 14 were thrombocytopenic.

3. Among the 21 patients with no bleeding, there were 10 with thrombocytopenia and 3 with increased numbers of platelets.

4. Evidence was presented in agreement with previous impressions that there is no constant relationship between the platelet count and the occurrence of bleeding. Within limits, the functional hemostatic activity of platelets appears to be more important than merely the absolute number.

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STUDIES ON SYNERGISM WITH TWELVE ANTIBIOTICS AGAINST THIRTY HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS

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MANY reports from hospitals in various parts of the world have indicated an increasing incidence of infections with antibiotic-resistant staphylococci. Therefore, combinations of 2 or more antibiotics are often used in treating staphylococcal infections to delay the emergence of resistant variants of staphylococci.

The antibacterial activity of antibiotics in pairs upon staphylococci has been studied to a limited extent. Rantz and Randall[1] reported the effect of 6 antibiotics in 15 combinations upon 33 strains of staphylococci in broth. They found that penicillin, streptomycin, and bacitracin were often "synergistic" when used together in pairs or added to chloramphenicol, oxytetracycline, or erythromycin. They defined synergism as killing within 24 hours with no secondary growth in subcultures, a phenomenon usually called the bactericidal effect.

Manten[2] found that streptomycin had a synergistic bactericidal effect with penicillin, chloramphenicol, tetracycline, or erythromycin against most of the 55 strains of staphylococci he tested. On the other hand, penicillin seemed antagonistic to any of the antibiotics except streptomycin. These effects were obtained by the use of antibiotic-impregnated paper disks on seeded agar plates and by observation of replica plates.

Kylin and Wall[3] studied the effect of penicillin and streptomycin upon the growth of 32 strains of staphylococci by the disk method. They found that these drugs often had a synergistic antibacterial effect. Waisbren[4] tested 8 antibiotics in pairs against 10 strains of staphylococci in broth with subcultures after 24 hours. He found no prevailing pattern of combined effects. Potentiation or interference with antibacterial activity between pairs of antibiotics was infrequent.

The purpose of the present investigation was to study the combined antibacterial activity of a larger number of antibiotics upon staphylococci isolated recently from patients in this hospital. The activity of the individual antibiotics was tested by the tube dilution method[5] with subcultures of each clear tube after 24 hours. The combined activity of 12 antibiotics in 66 pairs was tested against 30 strains of staphylococci by the paper strip-agar diffusion method of Elek[6] and Dye[7] with replica plating by the method of Lederberg.[8]

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Supported in part by a grant-in-aid from Eli Lilly & Co., Indianapolis, Ind.
Received for publication Jan. 7, 1957.

MATERIALS AND METHODS

Source of Cultures.—Thirty consecutive strains of hemolytic, coagulase-positive *Staphylococcus aureus* were isolated from patients in this hospital in late 1954. Nine were from blood cultures, 9 from skin lesions, 5 from urine, 3 from the nose or throat, 2 from body cavities, and 2 from wounds.

Tube Dilution Method.—Tube dilution antibiotic resistance tests were done on all 30 strains of staphylococci with 12 individual antibiotics.* Doubling dilutions of the antibiotics in nutrient broth (Difco) ranged from 100 units or mcg. to 0.39 unit or mcg. per milliliter. The bacterial inoculum was 0.5 ml. of a 1:100 dilution of an 18-hour broth culture and was mixed with 0.5 ml. of antibiotic in broth. The bacteriostatic end point was recorded as the lowest concentration of antibiotic that prevented visible growth after 18 hours at 37° C. All clear tubes were then subcultured by transferring one loopful with a 4 mm. inoculating loop to a nutrient agar plate. The lowest antibiotic concentration that prevented growth on subculture was recorded as the bactericidal end point. All strains showing no growth in a concentration of 6.25 units or mcg. per milliliter were arbitrarily called susceptible. Those showing growth at 6.25 units or mcg. per milliliter were recorded as resistant.

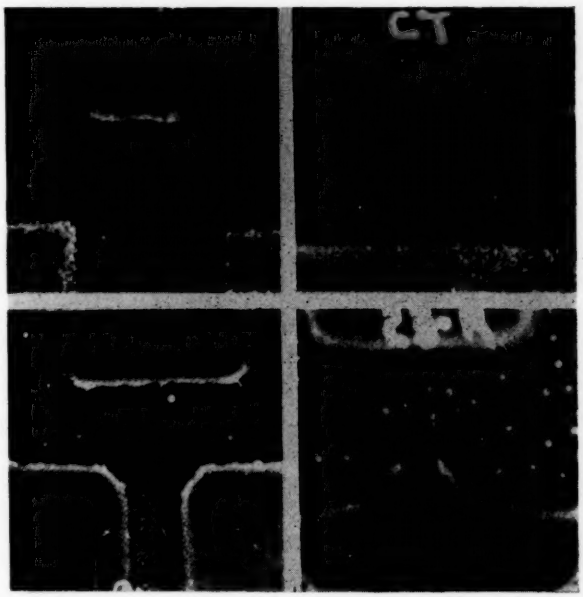

Fig. 1.—Growth inhibition patterns with paired antibiotics for staphylococci by paper strip–agar diffusion method: Upper left, no combined effect; upper right, addition; lower left, synergism; lower right, antagonism.

The Agar Diffusion Method.—Nutrient broth and agar (Difco) were used throughout the study. Imprint "stamps" for transplants to replica plates were prepared by fixing white velveteen to 1⅛ in. pine disks 80 mm. in diameter using polyvinyl acetate as the adhesive. These disks withstood cleaning and sterilizing at least one dozen times. Five per cent phenol solution was used to sterilize the "stamps" immediately after use. Standard 100 mm. Petri dishes were used for all plate work.

*The antibiotics were dissolved in sterile, distilled water. They included the potassium salt of benzyl penicillin G (Chas. Pfizer & Co.), dihydrostreptomycin sulfate (Merck & Co., Inc.), chlortetracycline hydrochloride (Lederle Laboratories), oxytetracycline hydrochloride (Chas. Pfizer & Co.), tetracycline hydrochloride (Chas. Pfizer & Co.), chloramphenicol (Parke, Davis & Co.), neomycin sulfate (The Upjohn Co.), bacitracin (The Upjohn Co.), erythromycin glucoheptonate (Eli Lilly & Co.), cycloserine (Eli Lilly & Co.), vancomycin (Eli Lilly & Co.), and novobiocin sodium (The Upjohn Co.) Solutions of chloramphenicol and the tetracycline group were prepared freshly. The other antibiotic solutions were stored at −15° C. for not more than one week.

White blotting paper (substance 160) was cut into strips 6 mm. wide by 30 mm. or 75 mm. long. These strips were sterilized, dried, and placed in a fresh aqueous solution of antibiotic. The excess fluid was poured off and the strips were partially dried one hour at 37° C. They were used immediately.* The concentration of the antibiotics in the strips was adjusted for each bacterial strain to give a zone of growth inhibition 2 to 7 mm. wide around the strips.†

Plates containing 15 ml. of agar were inoculated with 0.5 ml. of a 1:100 dilution of an 18-hour broth culture and incubated at 37° C. for one hour (until the surface appeared dry). One paper strip (6 × 75 mm.) containing an antibiotic was then placed across the diameter of the plate. Three shorter strips containing other antibiotics were placed at right angles and touching the long strip. The plate was incubated 18 hours. The zones of growth inhibition were measured and the bacteriostatic growth pattern at the junction of the antibiotic strips was recorded photographically.‡ These were then interpreted as showing no combined effect (right angle), addition (rounded corner), synergism (corner cut off straight), or antagonism (tongue of growth) as shown in Fig. 1. The paper strips were then removed and an imprint of the growth on this original plate was made with a velveteen "stamp" and transferred to a sterile agar replica plate. This replica plate was incubated 18 hours at 37° C. The zones of growth inhibition were measured and the bactericidal growth patterns at the junction of the antibiotic strips were recorded.

RESULTS

The bacteriostatic susceptibility of the 30 strains to 12 antibiotics is given in Table I. There was a high incidence of strains resistant to the antibiotics commonly used prior to 1954 (penicillin, streptomycin, and the tetracyclines). Bacteriostasis was achieved at the lowest average concentration by novobiocin, vancomycin, and erythromycin. Table II represents the bactericidal activity of the same 12 antibiotics. The most active bactericidal antibiotics were vancomycin, neomycin, and bacitracin.

Table III reveals the action of 12 antibiotics in all the possible paired combinations by the agar diffusion method. The cases with no combined effect are not shown and represent the difference of the totals from 100 per cent. The antibiotics are arranged in order of decreasing addition of bacteriostatic activity. Combination of 2 antibiotics usually showed no potentiation if the staphylococci were resistant to one or both antibiotics. Thus the incidence of addition was low with streptomycin, penicillin, and the tetracycline group. Vancomycin, neomycin, and streptomycin showed greater additive potentiation of bactericidal activity than bacteriostatic activity. True synergism was infrequent with any drug combination and antagonism was rare. Bacteriostatic antibiotics did not antagonize the antibacterial action of the bactericidal antibiotics. Penicillin and streptomycin had no synergistic activity against these rather resistant strains of staphylococci.

*Various methods of preparing antibiotic strips in advance were tried. The best method was to freeze them at −5° C. immediately after pouring off the excess fluid. Attempts at drying at room temperature, in an incubator at 37° C., and by quick-freezing at −70° C. with drying under vacuum at room temperature all resulted in considerable loss of activity of some of the antibiotics.

†The concentration of the antibiotics per milliliter was as follows: penicillin 5, 10, 20, or 30 units; streptomycin 100 or 200 mcg.; the tetracycline group 100 or 200 mcg.; chloramphenicol 50 or 100 mcg.; neomycin 2 mg.; bacitracin 20 units; erythromycin 100 or 200 mcg.; cycloserine 200 mcg.; vancomycin and novobiocin 100 mcg.

‡The plate was exposed on Repro-Negative Kodagraph Extra Thin Paper, Eastman Kodak Co., Rochester, N. Y., with a photoflood lamp. The paper was then developed, dried, and mounted.

TABLE I. THE BACTERIOSTATIC ACTIVITY OF 12 ANTIBIOTICS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (TUBE METHOD)

	CONCENTRATION IN UNITS OR MCG. PER MILLILITER									
	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	>100
Novobiocin	28*	1		1						
Vancomycin	17	11	1	1						
Erythromycin	3	16	5	1	4					1
Chloramphenicol		8	14	7		1				
Bacitracin	1	2	4	23						
Neomycin	5	2	2	13	8					
Cycloserine				1	25	4				
Oxytetracycline	10				5	1	1	12		1
Chlortetracycline	10			1	3	2		5	9	
Tetracycline	9		1		1	2	6	5	3	3
Streptomycin	2	2	7	1					1	17
Penicillin	10								3	17

*Number of strains whose growth was suppressed. Median point enclosed by square.

TABLE II. THE BACTERICIDAL ACTIVITY OF 12 ANTIBIOTICS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (TUBE DILUTION METHOD WITH SUBCULTURE)

	CONCENTRATION IN UNITS OR MCG. PER MILLILITER									
	0.39	0.78	1.56	3.125	6.25	12.5	25	50	100	>100
Vancomycin	6*	3	4	9	1	2	2	1	2	
Neomycin	4	1	2	13	5	2				
Bacitracin	1		1	8	8	7	3	1		1
Erythromycin	2	1	1	3	1	10	5	2	1	4
Novobiocin	5				2	3	3	6	11	
Oxytetracycline	1			1	4	4	4	2	2	13
Tetracycline	1		1	1	3	4		2	3	15
Chlortetracycline	1			1				3	16	19
Streptomycin	2	2		1	1	2	1	2		19
Penicillin	4		1			3	2		1	19
Cycloserine					4	1		2	2	21
Chloramphenicol		2	1					1	1	25

*Number of strains killed. Median point enclosed by square.

TABLE III. ACTION OF 12 ANTIBIOTICS IN PAIRS ON 30 STRAINS OF STAPHYLOCOCCI (PLATE METHOD)

	PER CENT ADDITIVE		PER CENT SYNERGISTIC		PER CENT ANTAGONISTIC	
	BACTERIOSTATIC	BACTERICIDAL	BACTERIOSTATIC	BACTERICIDAL	BACTERIOSTATIC	BACTERICIDAL
Cycloserine	55.2	50.3	7.6	8.8	0.6	1.2
Chloramphenicol	53.9	53.0	2.7	4.5	0.6	1.8
Bacitracin	52.4	51.2	4.5	6.1	0.9	1.2
Vancomycin	51.2	68.2	7.0	5.8	0.6	0.9
Erythromycin	47.3	49.7	4.2	6.1	0.3	3.3
Novobiocin	43.6	41.2	5.8	5.5	1.8	7.3
Chlortetracycline	42.7	35.2	2.4	6.1	0.3	1.5
Penicillin	36.7	36.7	4.8	5.8	0.9	2.7
Neomycin	34.2	48.5	2.4	4.8	0.9	0.9
Tetracycline	33.3	36.1	4.2	6.7	1.5	3.3
Oxytetracycline	30.6	31.2	2.7	3.6	0.6	1.5
Streptomycin	22.1	32.1	2.4	5.5	0.6	0.9
Average	41.9	44.5	4.2	5.8	0.8	2.2

Table IV shows the incidence of addition of both the bacteriostatic and the bactericidal activities of 66 paired antibiotics against 30 strains of staphylococci.

TABLE IV. INCIDENCE OF ADDITION OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF 12 ANTIBIOTICS IN PAIRS AGAINST 30 STRAINS OF STAPHYLOCOCCI

BACTERIOSTATIC		BACTERICIDAL												
		C	B	Cy	E	N	No	P	T	CT	S	OT		
		23	20	25	14	16	30	17	19	20	22	19	V	
C	10		27	7	24	22	10	10	14	15	13	10	C	
B	22	27		21	23	16	11	15	11	10	8	7	B	
V	22	20	18		22	23	10	14	11	13	7	13	Cy	
E	23	25	21	9		15	6	8	15	16	9	12	E	
No	13	19	13	17	17		26	9	9	7	12	5	N	
CT	15	15	15	23	15	11		14	7	5	11	6	No	
P	15	12	16	10	12	12	10		10	8	9	7	P	
N	25	18	14	9	6	13	8	9		8	6	9	T	
T	12	15	10	14	15	9	9	9	4		4	10	CT	
OT	15	10	9	16	11	8	10	8	3	9		5	S	
S	10	7	8	11	2	12	5	8	4	4	2			
	Cy	C	B	V	E	No	CT	P	N	T	OT			

BACTERIOSTATIC

Key to symbols: Cy—cycloserine, C—chloramphenicol, B—bacitracin, V—vancomycin, E—erythromycin, No—novobiocin, CT—chlortetracycline, P—penicillin, N—neomycin, T—tetracycline, OT—oxytetracycline, S—streptomycin.

The numbers represent the number of strains of staphylococci that showed addition with a pair of antibiotics. Note that the total number in Tables IV, V, and VII is usually less than 30; the difference equals the number of cases with no effect.

TABLE V. INCIDENCE OF SYNERGISM OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF 12 ANTIBIOTICS IN PAIRS AGAINST 30 STRAINS OF STAPHYLOCOCCI

BACTERIOSTATIC		BACTERICIDAL												
		T	E	B	CT	V	P	No	S	N	C	OT		
		3	6	1	2	2	1	5	3	1	3	2	Cy	
V	2		3	3	0	4	1	1	3	3	1	0	T	
No	6	0		0	1	0	3	0	2	2	1	2	E	
P	2	3	7		4	3	4	4	1	0	2	2	B	
B	1	3	4	0		3	2	0	4	4	0	0	CT	
E	5	0	0	1	1		3	0	0	0	2	2	V	
T	4	2	2	0	2	3		6	0	0	3	0	P	
C	0	2	0	3	2	1	1		0	1	1	0	No	
OT	1	5	0	0	1	2	0	0		2	1	2	S	
CT	3	1	0	0	0	1	0	0	0		1	2	N	
N	1	1	0	0	1	0	0	0	0	3		0	C	
S	0	4	0	0	0	0	0	0	0	2	2			
	Cy	V	No	P	B	E	T	C	OT	CT	N			

BACTERIOSTATIC

See Table IV for key to symbols.

Table V is a summary of the incidence of synergism of both the bacteriostatic and the bactericidal activities of all the possible combinations of the 12 antibiotics in pairs. The incidence of synergism was not as frequent as has been reported by others.[1-3] This may be the result of our more exact definition of synergism as well as the relatively high resistance of our hospital strains of staphylococci to many antibiotics.

Table VI gives a total of the data from Tables IV and V and shows a closer agreement with the high incidence of "synergistic" activity (potentiation) found by others.

TABLE VI. INCIDENCE OF ADDITION OR SYNERGISM OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF 12 ANTIBIOTICS IN PAIRS AGAINST 30 STRAINS OF STAPHYLOCOCCI

BACTERIOSTATIC			BACTERICIDAL												BACTERICIDAL
			C	B	Cy	E	N	No	P	T	CT	S	OT		
			25	23	27	14	16	30	20	23	23	22	21	V	
	C	10		29	10	25	23	11	13	15	15	14	10	C	
	B	23	29		22	23	16	15	15	14	14	9	9	B	
	V	24	22	21		28	24	15	15	14	15	10	15	Cy	
	E	28	26	22	9		17	6	11	18	17	11	14	E	
	No	19	19	17	17	17		27	9	12	11	14	7	N	
	CT	18	15	15	24	16	11		20	8	5	11	6	No	
	P	17	15	16	13	13	19	10		11	10	9	7	P	
	N	26	18	15	10	6	13	11	9		8	9	9	T	
	T	16	16	12	16	18	11	9	9	4		8	10	CT	
	OT	16	10	10	21	13	8	10	8	3	9		7	S	
	S	10	7	8	15	2	12	7	8	6	4	2			
		Cy	C	B	V	E	No	CT	P	N	T	OT			
	BACTERIOSTATIC														

See Table IV for key to symbols.

TABLE VII. INCIDENCE OF ANTAGONISM OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF 12 ANTIBIOTICS IN PAIRS AGAINST 30 STRAINS OF STAPHYLOCOCCI

BACTERIOSTATIC			BACTERICIDAL												BACTERICIDAL
			E	T	P	C	OT	CT	Cy	B	V	N	S		
			6	5	1	4	2	2	0	1	0	0	3	No	
	T	1		1	3	0	0	0	0	1	0	0	0	E	
	B	1	1		1	1	0	0	2	1	0	0	0	T	
	P	1	0	0		0	2	0	0	0	1	1	0	P	
	N	1	1	1	0		0	0	0	0	0	1	0	C	
	Cy	0	1	0	0	0		0	1	0	0	0	0	OT	
	C	1	1	0	0	0	0		0	0	2	1	0	CT	
	V	0	0	0	1	0	0	0		1	0	0	0	Cy	
	OT	0	0	0	0	0	1	0	0		0	0	0	B	
	S	1	0	0	0	0	0	0	0	1		0	0	V	
	E	0	0	0	1	0	0	0	0	0	0		0	N	
	CT	0	0	0	0	0	0	0	1	0	0	0			
		No	T	B	P	N	Cy	C	V	OT	S	E			
	BACTERIOSTATIC														

See Table IV for key to symbols.

Table VII presents the incidence of antagonism of antibacterial activity, both bacteriostatic and bactericidal. The incidence of antagonism was generally very low. It was most frequent when the bactericidal activity of novobiocin combined with erythromycin, tetracycline, or chloramphenicol was measured.

DISCUSSION

As shown in Table I only 10 of the 30 strains of staphylococci were susceptible to penicillin. Cross-resistance to streptomycin occurred with 80 per cent of

the penicillin-resistant strains and 20 per cent of the penicillin-susceptible strains. Cross-resistance to chlortetracycline, oxytetracycline, and tetracycline occurred in 20 per cent of the penicillin-susceptible and 70 per cent of the penicillin-resistant strains. Cross-resistance between the 3 antibiotics in the tetracycline group occurred in all but one resistant strain.

The combined activity of any two antibiotics usually was not enhanced if the strain of staphylococci was highly resistant to one or both antibiotics. Therefore synergism was infrequent between penicillin, streptomycin, and the tetracycline group. As yet resistance to the other antibiotics is not frequent, chiefly because they have not been widely used. The ability of staphylococci to become resistant to the new antibiotics has been proved in this laboratory and others.[9, 10] The value of new antibiotics in combinations probably will diminish as resistant strains of staphylococci increase.

In the case of those antibiotics to which the 30 strains of staphylococci showed a wide range of resistance, the broth dilution and agar diffusion methods of determining resistance generally showed a fairly good correlation. When the range of resistance was small, the two methods often gave results that were not in close agreement.

The present study indicates that antibiotic combinations in general may be expected to have greater antibacterial activity against most hospital strains of staphylococci than one antibiotic alone. The potentiation is usually only additive and not truly synergistic. The use of combinations of antibiotics in treating staphylococcal infections seems to delay the emergence of resistant strains of the organism.[10] One need not fear that bacteriostatic antibiotics will interfere with bactericidal antibiotics.

The results shown in Table VI indicate that the most frequent enhancement of the antibacterial activity of antibiotics in pairs occurs with the newer antibiotics, to which the hospital strains of staphylococci are most susceptible. These combinations include novobiocin with vancomycin, bacitracin with chloramphenicol, and erythromycin with cycloserine.

Many of the reports by others using paired antibiotics against staphylococci are not in close agreement with the results of this study. The probable reasons for this disagreement can be found in the use of different methods, in the varying definitions of synergism and in the fact that our study was done with relatively resistant strains such as are now common in most hospitals.

SUMMARY AND CONCLUSIONS

1. Thirty hospital strains of *Staphylococcus aureus* were exposed to 12 antibiotics, both singly and in 66 paired combinations. Antibacterial activity was measured by tube dilution with subcultures as well as by agar diffusion from paper strips with replica plating. The combined effect of the paired antibiotics was judged from the growth patterns around the paper strips.

2. A high degree of resistance to penicillin, streptomycin, and the tetracycline group was frequent. Cross-resistance was common to these antibiotics Resistance was rare to the newer antibiotics.

3. Bactericidal activity was most marked with neomycin, vancomycin, and bacitracin.

4. The 12 antibiotics in pairs rarely showed antagonism of bacteriostatic or bactericidal action. True synergism was seldom found with the accurate paper strip–agar diffusion method. Addition of both the bacteriostatic and the bactericidal activities was frequent with all strains of staphylococci susceptible to both antibiotics. Antibiotics to which the strains of staphylococci were highly resistant had little or no effect in a combination.

5. Enhancement of antibacterial activity against staphylococci was most frequent with the combinations of novobiocin with vancomycin, bacitracin with chloramphenicol, and erythromycin with cycloserine.

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SERIAL SERUM GLUTAMIC OXALACETIC TRANSAMINASE (SGOT) AND ELECTROPHORESIS STUDIES IN THORAZINE-INDUCED JAUNDICE

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THE use of Thorazine (chlorpromazine hydrochloride, 10-[γ-dimethylamino-propyl] 2-chlorphenothiazine hydrochloride) in a great variety of clinical conditions has become widespread. Certain side effects have come to light, such as drowsiness, nasal congestion, constipation, rash, postural hypotension, and paralysis agitans. Of perhaps more serious nature is an alteration in liver function.

A number of cases of jaundice following the use of Thorazine have been reported.[1-4] Studies of liver biopsies[5, 6] have suggested that the anatomic lesion is of the obstructive type, with centrilobular distention of bile canaliculi. Menguy and his associates,[7] from experimental work in dogs, have suggested that the cholestasis is due to increased resistance of the choledochoduodenal sphincter, related to the depressing effects of chlorpromazine on duodenal motility. The occurrence of eosinophils in the cellular exudate usually found has suggested that the reaction to the drug may be on an allergic basis. The anatomic lesion is not unlike that described in the rare case of jaundice following the use of cinchophen,[8] thiouracil,[9] or methyltestosterone.[10]

The problem of distinguishing the jaundice occurring during Thorazine therapy from extrahepatic obstructive jaundice may be a difficult one. Liver function tests have indicated a raised direct and total bilirubin, and an increased alkaline phosphatase level. Recently, the determination of the amount of glutamic oxalacetic transaminase (GOT) in the blood serum has been proposed as a useful study in the differentiation of obstructive and cytotoxic jaundice.[11-13] Although in the jaundice of viral hepatitis or acute yellow atrophy exceedingly high values for SGOT are obtained, in obstructive jaundice the serum levels are increased only moderately. In a recent paper discussing SGOT changes in liver diseases,[14] it was stated in the conclusion that the serum levels were increased in Thorazine jaundice. Data were not reported.

RESULTS

We recently had the opportunity of following serially the chemical changes in the blood in 3 patients who became jaundiced in the course of therapy with Thorazine. We thought it might prove of value to report the findings in these cases.

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Received for publication Jan. 25, 1957.
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CASE 1.—A 34-year-old white woman had received 25 mg. of Thorazine 3 times daily since March 5, 1956, for a depressive state. On March 25, she consulted her physician because of nausea, malaise, and fever. She was not clinically jaundiced. Physical examination was entirely negative. She was given tetracycline in doses of 250 mg. every 6 hours. Two days later, the patient returned, complaining of weakness and anorexia. Jaundice was then evident. Neither the liver nor the spleen were palpable. Thorazine was discontinued; she was placed on a high protein, high carbohydrate diet supplemented with large amounts of vitamins. At no time were the stools acholic. At the height of her hyperbilirubinemia, the urine contained bile, and test for urobilinogen was positive at a dilution of 1:160. Pruritus was minimal. By April 16, the patient had made a clinical recovery.

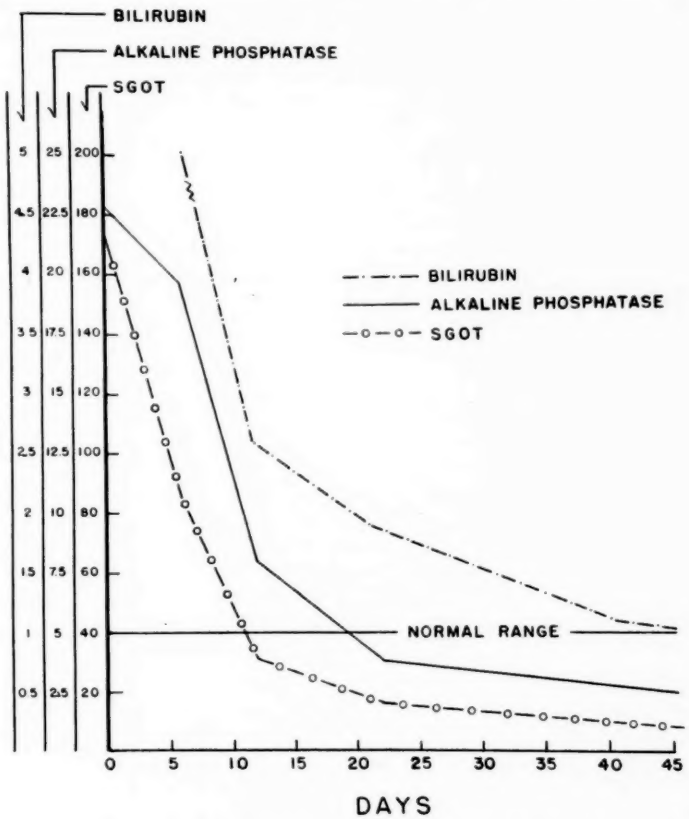

Fig. 1.—Case 1. Serial determination of bilirubin, alkaline phosphate, and SGOT.

The alterations in liver function are presented in Table I. The increases in bilirubin, serum alkaline phosphatase, and serum transaminase were maximal when determined 2 days after the clinical appearance of jaundice. The transaminase level was in the range that has been reported in obstructive jaundice; it returned to normal while the bilirubin and alkaline phosphatase were still significantly elevated (see Fig. 1). The thymol turbidity was never elevated, while the cephalin flocculation showed an insignificant rise at first, and then a slight increase when the bilirubin and enzyme levels had returned to normal. At all times the test for heterophil antibodies was negative. The chemical determination of blood proteins showed no significant abnormality. By paper electrophoresis, however, an inversion of the albumin-globulin ratio was found, which was not reversed until the other liver function tests had returned to normal. The inversion was due largely to a

decrease in albumin and an increase in the beta and gamma globulin fractions. This discrepancy between chemical and paper electrophoretic determinations of the albumin-globulin ratio has been observed by us before in cases of liver disease.[15]

TABLE I

SPECIMEN DATE	DAYS	BILIRUBIN (MG./100 ML.) DIRECT	BILIRUBIN (MG./100 ML.) TOTAL	ALKALINE PHOSPHATASE*	SGOT (UNITS/ML.)	TOTAL PROTEIN (GM./100 ML.)	CHEMICAL ALBUMIN-GLOBULIN RATIO	ELECTROPHORETIC PROTEIN PARTITION (PER CENT) ALBUMIN	GLOBULINS α_1	GLOBULINS α_2	GLOBULINS β	GLOBULINS γ
3/28/56	0	9.8	14.7	22.9	175	7.5	4.5/3.0	47.5	4.8	8.6	15.1	24.0
4/ 3/56	6	2.7	5.2	19.6	85	7.0	4.2/2.8	48.3	6.3	10.7	16.6	18.1
4/ 9/56	12	1.6	2.6	8.0	31	6.3	4.1/2.2	48.7	4.7	10.1	15.5	21.0
4/19/56	22	0.8	1.9	3.9	17	6.3	4.1/2.2	53.3	3.6	9.5	13.9	19.7
5/ 8/56	41	0.4	1.1	2.8	10	6.2	4.3/1.9	64.2	4.2	6.7	10.0	15.0
5/24/56	55	0.2	1.0	---	9	6.1	4.2/1.9	67.2	2.7	6.2	9.7	14.2

*Bodansky units per 100 ml.

CASE 2.—A 52-year-old white man had been troubled by cough for several months. In March, 1956, he was bronchoscoped. Various therapeutic procedures brought no relief. On July 17, he saw an otolaryngologist, who prescribed Thorazine 3 times daily. On July 25,

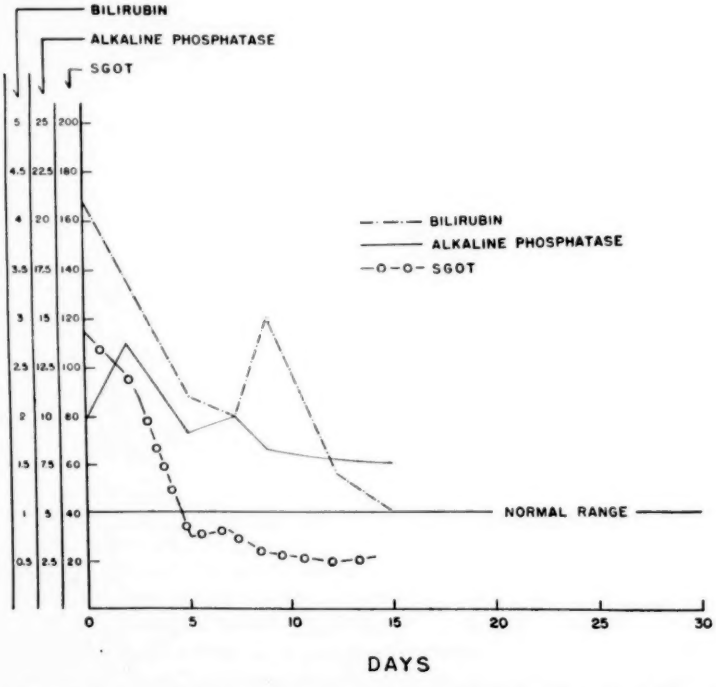

Fig. 2.—Case 2. Serial determination of bilirubin, alkaline phosphate, and SGOT.

he felt weak and tired. The following day, he had a fever of 103° F., and noted that his urine was dark. Five days later, he was hospitalized because of scleral icterus. His liver was palpable one centimeter below the right costal margin, and was tender. Blood count showed a slight eosinophilia. His bilirubin and alkaline phosphatase were elevated, as

was his serum transaminase. Thymol turbidity and cephalin flocculation were not significantly disturbed during the entire period of observation. At all times the test for heterophil antibodies was negative. In addition, there was no significant alteration in his blood proteins as measured chemically or by electrophoretic partition. On the fourth hospital day, a liver biopsy was done. The sections showed a mononuclear and slight eosinophilic infiltration in portal areas, and focally in the parenchyma. No focal necroses were found, but an occasional mitotic figure was seen. Centrally there was bile stasis, with plugging of canaliculi. The histologic picture was interpreted as being consistent with Thorazine hepatitis. On the sixth day after admission his SGOT was already in the normal range, although the bilirubin and alkaline phosphatase values were still elevated. Clinically, his serum bilirubin gradually decreased, although 2 weeks after admission to the hospital, it was still elevated. (Table II.)

TABLE II

SPECIMEN DATE	DAYS	BILIRUBIN (MG./100 ML.) DIRECT	BILIRUBIN (MG./100 ML.) TOTAL	ALKALINE PHOSPHATASE*	SGOT (UNITS/ML.)	TOTAL PROTEIN (GM./100 ML.)	CHEMICAL ALBUMIN-GLOBULIN RATIO	ELECTROPHORETIC PROTEIN PARTITION (PER CENT) ALBUMIN	GLOBULINS α_1	GLOBULINS α_2	GLOBULINS β	GLOBULINS γ
8/ 1/56	0	2.6	4.2	9.9	115	6.0	3.5/2.5	57.3	4.9	8.5	13.4	15.9
8/ 3/56	2	1.8	3.4	13.2	95	6.7	3.7/3.0	59.0	2.4	9.3	13.2	16.1
8/ 6/56	5	1.3	2.2	9.4	30	6.7	3.7/3.0	59.2	2.5	8.0	13.9	16.4
8/ 8/56	7	1.0	2.0	10.0	33	6.7	3.7/3.0	60.6	2.4	7.3	14.5	15.2
8/10/56	9	1.1	3.0	8.2	24	6.6	3.7/2.9	60.7	2.0	6.5	12.4	18.7
8/13/56	12	0.8	1.4	7.6	20	6.7	3.9/2.8	63.0	2.7	6.2	13.0	15.1
8/16/56	15	0.6	1.0	7.5	23	6.4	3.6/2.8	61.0	2.5	7.6	12.6	16.3

*Bodansky units per 100 ml.

CASE 3.—A 27-year-old American-born white woman of Mediterranean ancestry had a long history of abdominal pain, probably related to gynecologic disorders. She was admitted to this hospital on July 19 because of jaundice and right upper quadrant pain. An obstructing calculus was suspected by the admitting physician. Some weeks before, at another hospital, she had been given Thorazine. The exact amount was not known. Her jaundice had been first noted 10 days before her admission with developing pruritus, and lightening in color of her stools. On admission, her liver was palpable 3 fingers below the costal margin, and her spleen was also palpable. Her initial blood chemistry studies showed elevated bilirubin, alkaline phosphatase, and SGOT. Unfortunately, no data are available for the

TABLE III

SPECIMEN DATE	DAYS	BILIRUBIN (MG./100 ML.) DIRECT	BILIRUBIN (MG./100 ML.) TOTAL	ALKALINE PHOSPHATASE*	SGOT (UNITS/ML.)	TOTAL PROTEIN (GM./100 ML.)	CHEMICAL ALBUMIN-GLOBULIN RATIO	ELECTROPHORETIC PROTEIN PARTITION (PER CENT) ALBUMIN	GLOBULINS α_1	GLOBULINS α_2	GLOBULINS β	GLOBULINS γ
7/20/56	0	3.6	6.1	---	120	---	-----	---	---	---	---	---
7/23/56	3	---	---	22.2	---	---	-----	---	---	---	---	---
7/30/56	10	1.6	2.5	11.6	104	7.3	3.7/3.6	52.4	3.2	8.9	12.9	22.6
8/ 2/56	13	0.9	1.5	10.4	67	6.6	3.4/3.2	52.0	3.2	8.8	12.0	24.0
8/ 6/56	17	0.6	1.1	8.0	50	6.8	3.6/3.2	52.8	3.2	7.2	12.8	24.0
8/ 8/56	19	0.5	0.7	7.0	33	7.1	3.6/3.5	53.9	2.6	7.7	12.8	23.0
8/10/56	21	0.4	0.6	7.9	25	6.7	3.6/3.1	52.3	3.9	8.4	12.2	23.2
8/13/56	24	0.4	0.6	5.7	22	7.0	3.5/3.5	54.2	2.3	7.4	14.1	22.0
8/16/56	27	0.3	0.4	7.2	25	7.1	3.6/3.5	54.8	3.0	6.8	12.8	22.6

*Bodansky units per 100 ml.

first 10 days of the patient's jaundice. The bilirubin levels suggest that by the time this patient was admitted to the hospital, the jaundice was already decreasing. As examination of Table III and Fig. 3 indicates, the fall in bilirubin, alkaline phosphatase, and SGOT was more or less parallel, although the SGOT reached normal levels slightly before, and alkaline phosphatase slightly after, bilirubin. Cephalin flocculation, thymol turbidity, serum cholesterol, and heterophil antibody reaction were not significantly altered. There was slight elevation in Bromsulphalein dye (BSP) retention in a single determination at a time when SGOT, alkaline phosphatase, and bilirubin were still significantly elevated, though on the downgrade. Subsequently, the BSP returned to normal. Throughout the period of study, the gamma globulin was elevated.

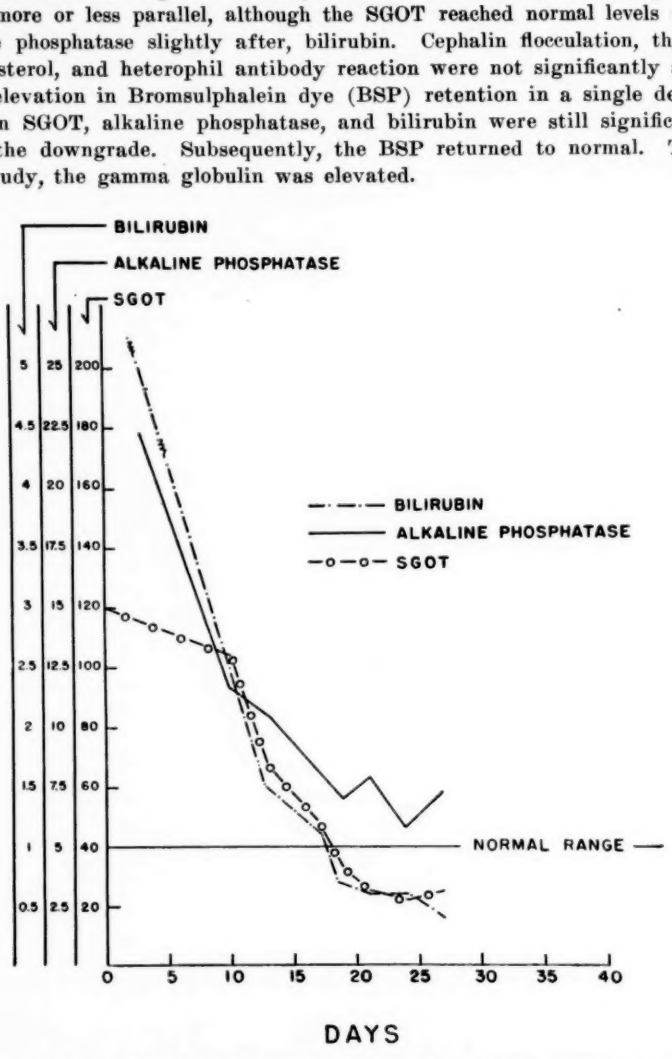

Fig. 3.—Case 3. Serial determination of bilirubin, alkaline phosphate, and SGOT.

On admission, a calculus obstructive jaundice was considered, but this became unlikely despite a nonfunctioning gall bladder on roentgenographic studies. One week after admission, she was transferred from the surgical to the medical service, where the differential diagnosis was considered to lie between infectious hepatitis of the cholangiolitic type and Thorazine hepatitis. The enlarged spleen was difficult to interpret. Studies of peripheral blood and bone marrow appeared to rule out a blood dyscrasia and hemolytic crisis. A liver needle biopsy, done on the twelfth hospital day, showed only pericentral cholestasis. No cellular infiltrate was found, and no necrosis. The histological picture gave no support to a diagnosis of hepatitis, of any origin, but, rather, was consistent with extrahepatic biliary obstruction. The patient developed an ascending urinary tract infection while in the hospital, for which she was treated. After almost one month in the hospital, she was discharged without definite diagnosis as to the nature of her jaundice.

DISCUSSION

In Cases 1 and 2, in which the diagnosis of Thorazine-induced hepatitis appears fairly certain, examination of Tables I and II and Figs. 1 and 2 shows the SGOT level moderately elevated shortly after the appearance of jaundice and falling off rapidly, with return to normal before the bilirubin or alkaline phosphatase values were again in the normal range. By contrast, Case 3 (Table III and Fig. 3) still showed an elevated SGOT some weeks after jaundice developed, a flatter type of curve, with more gradual return to normal, and closer correspondence with the curves for bilirubin and alkaline phosphatase levels. It is unfortunate that it was not possible to have data during the period of early jaundice, so that the entire curve could have been plotted. It would have been important to know the peak of SGOT. Although the clinical diagnosis of Thorazine hepatitis was never discarded in this case, it does not appear a likely one. Certainly, the liver biopsy lends no support. The behavior of the SGOT differs in this case from the other two. It would, doubtless, be desirable to have many more cases of Thorazine-induced jaundice to establish a pattern. If that demonstrated by the first 2 cases were consistently found, i.e., sharp fall of a moderately elevated SGOT, it would prove an excellent and invaluable tool in establishing the diagnosis of Thorazine-induced hepatitis. It is hoped that this report will stimulate others to obtain such information.

The addition of SGOT determination to the usual battery of liver function tests may prove of great value in helping to determine if jaundice developing in the course of Thorazine therapy is related to the drug, or to coincidental extrahepatic obstruction. The rapid return of SGOT titer to normal appears to be similar to that described following acute myocardial infarction, and to that seen due to the toxic effect of drugs, e.g., carbon tetrachloride,[11, 12] and heavy metals upon the liver. The dissociation of the temporal curves for SGOT, and alkaline phosphatase and bilirubin may prove of great value in the differential diagnosis.

SUMMARY

Three cases of jaundice developing in the course of Thorazine therapy were followed by serial determinations of serum glutamic oxalacetic transaminase, blood protein fractions by paper electrophoresis, serum bilirubin, and alkaline phosphatase. Of possible diagnostic help was the finding in cases of Thorazine-induced jaundice of a moderate rise in SGOT level, and its rapid return to normal while the bilirubin and blood alkaline phosphatase levels were still significantly increased.

We wish to express our appreciation to Drs. J. Schley, S. Siegel, and E. B. LeWinn for the clinical data in their cases.

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EXPERIENCES IN HUMAN BEINGS WITH AN IMPROVED FAT EMULSION FOR INTRAVENOUS ADMINISTRATION

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INTRODUCTION

THE need for a parenteral solution of high calorigenic value is well accepted. The increased interest in geriatric problems, as well as the extensive surgery that is possible today, makes the perfection of such a preparation even more urgent. However, the very fact that there is not available on the market today a fat emulsion for intravenous use emphasizes the problems in its production on a commercial basis. These problems have centered about the physical stability of the emulsion and adverse reactions to it in the recipient.

Numerous reports have appeared in the medical literature concerning both the preparation of various types of fat emulsions, as well as their use in animals and humans. It is not my purpose to review this literature; however, the interested reader is referred to the excellent monograph by Freeman[1] on this subject. The purpose of the present paper is to report our experiences with an improved fat emulsion prepared by The Upjohn Company† and contrast it to our previous experiences with a less satisfactory, but similar, product prepared by the same company. Since recent large scale clinical reports have appeared with somewhat conflicting data on reaction rates utilizing different types of emulsions,[2, 3] it is felt that this report will supply further information on which an appraisal of the practicability of such a product can be based.

METHODS

The subjects infused with the fat emulsion were a heterogenous group of hospitalized patients on the wards of the Colorado General Hospital, Denver General Hospital, and the Veterans Administration Hospital of Denver. A small number of normal volunteers were studied on the metabolic ward of the United States Army Medical Nutrition Laboratory. The majority of the hospitalized patients were on the Surgical Service and represent a great variety of surgical and medical problems. They were infused sometimes before and sometimes after surgery. No attempt was made to select patients, either according to disease or to severity of illness, except for 6 cirrhotic patients. In many instances the patients were receiving other intravenous fluids, as well as various medications. No attempt was made to alter the pre-existing medical regimen. The fat emulsion

From the U. S. Army Medical Nutrition Laboratory.

The opinions expressed in this paper are those of the author and do not necessarily represent those of any governmental agency.

Received for publication Jan. 28, 1957.

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†The author wishes to thank Drs. E. A. Hawk and R. W. Heinle of The Upjohn Co., Kalamazoo, Michigan, for their generosity in furnishing the fat emulsion, Lipomul.

was given merely as an adjuvant. Many subjects had more than one infusion, although the majority received only one, since we were primarily interested in accumulating data on reaction rates. Only in special instances was the material given for therapeutic purposes over a long period of time.

The fat emulsion, Lipomul, contained 15 per cent cottonseed oil, 4 per cent glucose, 1.2 per cent soybean phosphatide, and 0.3 per cent Pluronic F 68. It was bottled in 600 ml. quantities which, according to the formula, provided approximately 900 calories per bottle. Since comparison will be made between an improved emulsion and an older one, reference will be made to the "old" emulsion and the "new" emulsion. The formulation of the 2 emulsions is precisely the same. The difference between the two resides in the purification of the soybean phosphatide incorporated into the new emulsion.

Although minor variations are unavoidable when studying such a hospital population, an attempt was made to standardize the actual administration of the emulsion as much as possible. The infusion was always started with a solution of 5 per cent dextrose dissolved in water. The emulsified fat was then admitted to the system via a needle in the rubber adapter. If other fluids were being given, the fat was preceded and followed for an hour by 5 per cent dextrose and water. Saline was carefully avoided in the system to prevent "breaking" of the emulsion which occurs in the (in vitro) presence of electrolytes. A total of 600 ml. of emulsion were given over a 3½-hour period, starting at a rate of 8 to 16 drops per minute for 20 minutes. If no reaction occurred, the rate was increased to 36 drops per minute for another 20 minutes. If the clinical condition at the end of this period of time permitted, the rate was increased to 68 drops per minute for the remainder of the infusion. Body temperature, pulse, blood pressure, and respirations were measured at 20-minute intervals for the hour before and the hour after the institution of the infusion. Thereafter, these vital determinations were performed at hourly intervals for the next 11 hours. A physician was in attendance throughout the entire infusion in each case.

When an adverse reaction occurred, the infusion was immediately stopped. If the situation demanded, appropriate medications were administered. In the great majority of instances, simply stopping the fat sufficed and reinstitution of the infusion was possible.

TABLE I. CHART DEPICTING REACTION RATES USING THE "OLD" AND "NEW" FAT EMULSION FOR INTRAVENOUS ADMINISTRATION ACCORDING TO NUMBER OF SUBJECTS AND NUMBER OF INFUSIONS

EMULSION	OLD (AUG., 1954–DEC., 1955)*				NEW (JAN., 1956–OCT., 1956)			
Number of Infusions	293				362			
Number of Subjects	110				151			
REACTIONS	NUMBER OF INFUSIONS	PER CENT PER INFUSION	NUMBER OF SUBJECTS	PER CENT PER SUBJECT	NUMBER OF INFUSIONS	PER CENT PER INFUSION	NUMBER OF SUBJECTS	PER CENT PER SUBJECT
Fever†	45	15	32	29	60	17	45	29
Chill	21	7	18	16	2	.6	2	1.3
Back and chest pain	38	13	33	30	0	0	0	0
Flushing	7	2	7	6	1	.3	1	.7
Nausea and vomiting	15	5	14	13	4	1	4	2.6
B.P. rise and fall	20	7	14	13	0	0	0	0
Dyspnea cyanosis	14	5	14	13	0	0	0	0
Shock	2	0.7	2	2	0	0	0	0
Rash	0	0	0	0	1	.3	1	.3
Miscellaneous (twitching, itching, hives, abdom. cramps)	6	2	6	5	3	.8	3	2
Over-all				51				33

*The author is indebted to Dr. Hugo Moeller and Dr. Morton Grossman for some of the data on the "old" emulsion used in this report.

†Over 100.6 orally or 101.6 rectally.

RESULTS

Table I depicts the recorded observations with the 2 emulsions in a comparable number of subjects. It is clear that there is a striking decrease in the over-all reaction rates with the new emulsion as compared to the old one. This is further exemplified by the fact that only 3 of 362 infusions could not be completed with the new emulsion, as compared with 23 of 293 that could not be completed with the old emulsion. Since those infusions which were discontinued were complicated by severe reactions, most of these patients were not reinfused; this makes the above comparison even more striking.

The various types of reactions encountered during the intravenous administration of emulsified fat have been described adequately by others. Often an attempt has been made to categorize some of the reactions into groups, e.g., "colloid reaction," and "histamine reaction." For purposes of clarity and comparison, each sign or symptom will be discussed separately, although it is recognized that many of the reactions may well be on a common etiologic basis.

TYPE AND INCIDENCE OF REACTIONS

It will be noted in Table I that data are given for the various reactions according to number of infusions and number of patients. This indicates that some subjects received more than one infusion. The consistently lower value for per cent per infusion as compared to per cent per subject reflects the often noted phenomenon of tolerance. Thus, most reactions occur on the first infusion and are much less likely to occur during successive ones.

Fever.—A rise in bodily temperature above the preinfusion morning temperature occurred in over 95 per cent of our subjects. However, since diurnal rise in body temperature is a common occurrence, and we were studying primarily a group of hospitalized patients who might be expected to have some fever from their primary illness, it was necessary that a significant febrile reaction be defined. A rise in temperature of 2° F. was not acceptable since many of our subjects had a morning temperature of 97° F. and a rise to 99° F. certainly could not be considered an adverse reaction. Hence, it was arbitrarily decided that, to be considered a significant febrile reaction, the temperature must rise in the 12-hour observation period at least 2° F. over the accepted normal rectal temperature of 99.6° F., or at least 2° F. over the control temperature if that control temperature was above 99.6° F. rectally.

Using the above criteria, 29 per cent of the subjects developed fever as a consequence of the infusion of the old emulsion as compared to 29 per cent with the new emulsion. The per cent per infusion is 15 and 17, respectively, for the two preparations. The fact that there is little difference in the febrile reaction rate between the two emulsions is considered important and will be referred to later. The nature of the febrile reaction, however, did differ somewhat in the two instances. With the new preparation, all of the significant temperature rises occurred as a late manifestation, often 2 to 4 hours after the infusion had been completed. Although this was often true with the old

emulsion, 13 per cent of the febrile reactions did occur during the course of the infusion. However, the height of the temperature rise was not significantly different (Fig. 1).

It was interesting that, in most instances, the subjects were unaware of the fever except when it was accompanied by a chill or was markedly elevated.

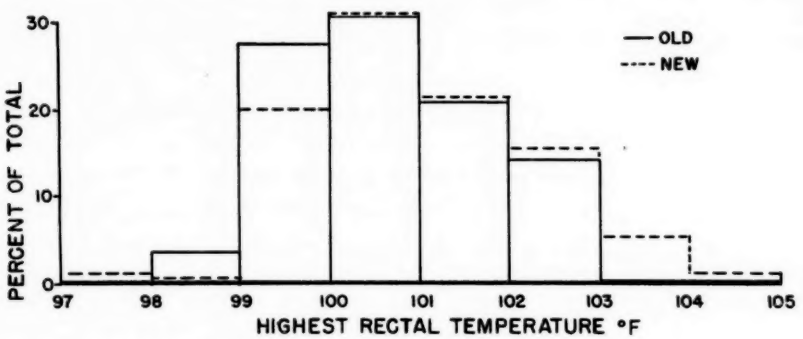

Fig. 1.—The incidence of the highest rectal temperature which occurred as a result of the first infusion of an "old" and "new" fat emulsion.

The duration of fever was variable but, for the most part, lasted only an hour or two. It was most common for the temperature to rise and fall rather rapidly.

Chill.—Sixteen per cent of the subjects with the old emulsion as compared to 1.3 per cent with the new one experienced chill as a complication of intravenous fat. The chill was delayed in the two subjects receiving the new emulsion.

Pain.—Back pain was the second most common reaction with the old material. It occurred in 30 per cent of the subjects and 13 per cent of the infusions. This type of reaction has not been observed in any of our patients receiving the new preparation. The reaction, when it occurred, was acute, often appearing after only a few drops of the material had been given and always within the first few minutes. Most subjects described it as a colicky or griping pain in the lumbosacral area. Rarely, it would occur across the chest or down the legs or arms. It was a frightening experience for the patient, but fortunately would subside within a few minutes after discontinuation of the infusion. No objective findings accompanied the pain and, as far as we know, no subsequent disability in any form occurred. Reinstitution of the infusion was possible in all instances without a recurrence of the symptom.

Flushing.—Six per cent of the subjects and 2 per cent of the total infusions were complicated by a flush with the old emulsion, while only one patient out of 151 experienced this phenomenon with the present one. Although uncomfortable to the subject, the flush disappeared promptly with temporary abandonment of the infusion.

Nausea and Vomiting.—This set of adverse reactions occurred in only 2.6 per cent of our subjects with the new preparation as contrasted to 13 per cent of the group receiving the other. Nausea and vomiting generally occurred

after the infusion had been completed, although on several occasions one or the other was the cause of permanent discontinuation of the fat. The effect of the intravenous fat on appetite was difficult to assess. Some patients would eat heartily immediately after completion of the injection, while others complained of anorexia. In the latter case, the appetite had always returned by the evening mealtime.

Blood Pressure Variations.—Again, arbitrary limits had to be placed on those variations in blood pressure which would be considered abnormal and, therefore, a reaction to intravenous fat. A rise of 40 mm. Hg systolic or 20 mm. Hg diastolic or a fall in systolic blood pressure of from 20 to 40 mm. Hg, depending upon the control level, was considered a reaction. Utilizing these criteria, 13 per cent of the subjects and 7 per cent of the infusions were complicated by significant blood pressure changes when the old emulsion was administered. There were no such changes observed with the new emulsion. Of all of the various reactions formerly observed, the rise in blood pressure seemed to be most closely related to rate of infusion and almost always could be lowered by decreasing the flow.

Dyspnea and Cyanosis.—The symptom-sign complex of dyspnea and cyanosis occurred in 13 per cent of the subjects and 5 per cent of the infusions when the old emulsion was administered. None of the patients were thus affected with the new preparation. These reactions occurred early in the course of the infusion and often were severe enough to prompt permanent discontinuation. Appropriate medications such as antihistamines, adrenalin, aminophylline, and oxygen were utilized on occasion as necessary. Although it would be difficult to state definitely which medicament, if any, was effective on such a small group of patients, prompt discontinuation of the emulsion undoubtedly was most important.

Shock.—A full-blown picture of anaphylactoid shock was seen in 2 of our patients receiving the old emulsion. Both reactions occurred within the first 3 minutes of the infusion. Fortunately, both patients survived without any obvious sequelae. However, there is no doubt that, without immediate attention in both instances, a dire outcome could have been expected. No such reactions have been encountered with the emulsion presently employed.

Rash.—This reaction had never been observed until recently in one child receiving the new emulsion. It was of the erythema multiforme variety, moderately pruritic, appearing after approximately 30 minutes of the injection time had elapsed. Attempts were made on 5 different occasions to administer the fat, but the rash appeared each time. Prompt subsidence of the erythema would follow discontinuation of the emulsion. Prior and simultaneous administration of intravenous antihistamines was ineffective. Plans to skin test the child with each ingredient of the emulsion were interrupted by discharge from the hospital.

Miscellaneous.—Two bizarre reactions were observed with the new preparation which require further mention. A 34-year-old woman with advanced Laennec's cirrhosis of the liver was infused on 4 different occasions. With

each infusion she complained of a nagging backache, which appeared toward the end or after the end of the infusion and lasted for several hours. The reaction was accompanied by gross tremor of the hands and head, some vomiting, and a moderate rise in temperature. When it occurred during the infusion, discontinuation of the fat had no effect. Past history revealed that the patient had made numerous visits to the receiving ward because of gross tremors of the body and had been admitted to the hospital on one occasion for hysterical paralysis of the legs. In this instance, an adverse reaction to the intravenous fat undoubtedly occurred; however, it was clouded by an overtone of hysteria. It is interesting to note that of the 11 patients with cirrhosis of the liver who were studied, this is the only reaction other than fever that occurred in the group.

The second patient was an 89-year-old man with severe generalized arteriosclerosis and arteriosclerotic heart disease. After 480 ml. of the infusion had been administered without mishap, 30 mg. of codeine sulfate were given for restlessness. Semistupor, accentuated by Cheyne-Stokes respiration, supervened within 30 minutes and the fat emulsion was discontinued. By late afternoon, the patient's condition had returned to that which was maintained prior to the infusion. In this instance the role of intravenous fat in the production of the semistupor is not clear.

In the total of 655 infusions there was no mortality and no permanent disability from any acute morbidity.

ATTEMPTS TO ALTER REACTION RATES

Interest in effecting a reduction in reaction rates was motivated not only from a therapeutic viewpoint but also in an effort to learn more about the mechanisms of the reactions. An insight into the latter might indicate directions to pursue in the production of a more suitable fat emulsion. Since most of the reactions occurred with the old emulsions, attempts to alter the reaction rates were performed in patients receiving that preparation.

Rate of Infusion.—Previous work in this laboratory in normal human volunteers showed that varying the time required for the infusion from an average of 131 minutes to 356 minutes did not affect the febrile response as measured by fever indices.[4] In the present study, an analysis of the correlation between the initial rate of infusion and the occurrence of back pain in 81 subjects revealed a negative value ($P > 0.50$). However, flow did seem to be correlated with elevation of blood pressure and since so many of the severe reactions, such as shock, dyspnea, or cyanosis, occurred early in the infusion period, we concur with others that all infusions of emulsified fat should be started slowly and the subject should be closely observed at the beginning of the injection.

Repeated Infusions.—It has been commonly observed that repeated infusions in the same person result in less frequent adverse reactions. This tolerance or tachyphylaxis was evident in our experience, although by no

means invariably so. That tolerance to the febrile reaction develops is evident in Fig. 2. However, one of the patients had an elevation of oral temperature above 102° F. on each of 6 successive infusions. Another subject had an elevation of rectal temperature to 101.6° F. only on the fifth day of successive infusions. The tolerance was more consistently observed in the case of back pain, flushing, nausea, and vomiting.

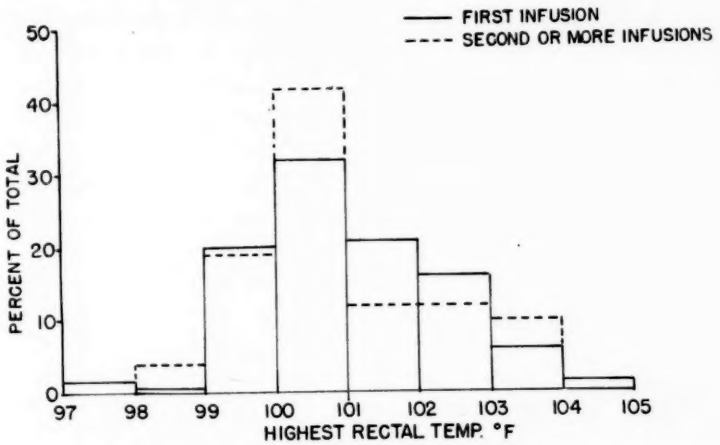

Fig. 2.—A distribution graph depicting the incidence of the highest temperature recorded during a 12-hour period following the intravenous infusion of 600 ml. Lipomul on the first occasion and succeeding ones.

Use of Antihistaminics.—Many of the immediate reactions resemble in some respects a histamine-like response. With this in mind, 14 patients were studied with infusions of intravenous fat one week apart. All patients received 50 mg. of Benadryl intravenously 15 minutes before and again intravenously halfway through one of two infusions. Nine of the 14 received Benadryl with their second infusion. In this small series of patients, wherein each subject serves as his own control, the incidence of reactions of all types was 35 per cent with Benadryl and 50 per cent without. The slight decrease in reaction rate was not considered significant enough to pursue further.

Addition of Serum Albumin.—Seventeen patients received an infusion of the emulsified fat to which had been added 18 ml. of a 25 per cent human serum albumin solution. Nine of these subjects were also infused a second time, one week apart, with an unsupplemented bottle of emulsion. Thirty per cent of the 17 patients experienced some type of reaction with the serum albumin-fat mixture, as compared to the over-all reaction rate of 51 per cent in the 110 subjects receiving the old emulsion. Of the 9 subjects who had 2 infusions, 3 developed reactions with the serum albumin-fat mixture and 4 had reactions with the regular material.

DISCUSSION

The remarkable reduction in adverse reactions, with the exception of fever, which occurred coincident with the purification of the soybean phosphatide used

as emulsifier in the new emulsion has been gratifying. The fact that, out of a total of 362 infusions, only 3 could not be completed, attests to its practicability and safety. The remarkable similarity in the incidence of fever between the old and new preparations suggests that the febrile response is something inherent in the administration of fat and has very little relation to impurities which presumably were removed during the process of purification of the phosphatide. These observations have led us to explore the relationship of the febrile response to the utilization of the fat, the details of which are the basis of a subsequent paper. Suffice it to say that there is a highly significant correlation between the rapidity of clearing of the infused fat from the blood and the febrile response. These data suggest that fever consequent to intravenous fat may result from rapid oxidation of the fat with resultant endogenous heat production. The mildness of the fever in the great majority of instances in our opinion is not a contraindication to the use of this preparation. Although careful metabolic studies on the effect of the delayed fever on nitrogen or caloric balance have not been done, we agree with Jordan[3] that the threat of a febrile reaction should not deter use of intravenous fat emulsion when indicated. We feel that the high incidence of fever in this report, as compared with other published results, reflects the plan of the experiment, namely, that the temperature recording was continued on an hourly basis 8½ hours after the infusion had been completed. The nature of the febrile response is such that it could be overlooked if the temperatures were recorded at the usual 4-hour intervals. Furthermore, if the rate of clearing has anything to do with the febrile response, then the rate of administration and, in particular, the grams of fat administered per unit of time may be critical. This is suggested by Kinsell's[5] observation that administration of more than 10 Gm. of fat per hour is attended by a significant increase in reaction rate. We administered approximately 25 Gm. of fat per hour, which may account for our higher incidence of fever. However, in our opinion prolonged administration is impractical, particularly in those patients requiring other intravenous fluids.

Since we were interested primarily in the incidence of adverse reactions to intravenous fat and because the phenomenon of tolerance was appreciated, the majority of our cases received only one infusion. Thus we have not been in a position to assess the beneficial effects of the preparation on a scientific basis in most patients. However, from a strictly clinical point of view, there is reason to believe that the material was helpful in those few patients who received it over a period of several days. This view is shared by those on the house staff who were disinterested observers. Likewise, no significant observations on the effect of the emulsified fat on laboratory data could be made because of the nature of the experiment. Others have shown both in animals and man that the infused fat is utilized for energy purposes, and improvement in negative balance as well as weight gain has been demonstrated in ill subjects.[1, 6, 7]

In attempting to evaluate what factor or factors are responsible for the adverse reactions, the host susceptibility must be considered. It is our experience, as well as that of others,[2] that the more severely ill patients tend to develop more reactions and more severe reactions than the less ill or normal subjects. The distinction between severity of illness is an extremely difficult one, based primarily on clinical judgment. One cannot use vital signs for this delineation, nor any known laboratory test. We tried to correlate reaction rate with preinfusion total serum protein and albumin-globulin ratio in 80 patients, but were unsuccessful. Geyer[8] has noticed that serum from severely ill patients will produce clumping of the emulsion in vitro, whereas serum from healthy persons will not. This observation has not been made in vivo. We were unable to confirm the opinion of others that patients with liver disease do not tolerate intravenous fat very well.

Protein has been shown to be an excellent stabilizer of fat emulsions and has been used as such by Shafiroff and co-workers.[9] Recent studies on the chemical composition of natural chylomicrons by others,[10, 11] as well as in our own laboratory, have shown that protein is an integral part of the fat particle. It was on the basis of this information that serum albumin was added to the emulsion and tested clinically. Although some reduction did occur in the reaction rate, the results did not compare with those using the new emulsion which, of course, is protein free. However, the addition of supplemental protein to the emulsion is still desirable since it would add appreciably to the nutritive value of the material. The present formula will not permit supplementation with amino acids, since the latter will "break" the emulsion, as does the addition of electrolytes. This is a flaw in the emulsion which, although not critical, is serious enough to make imperative the search for a better emulsion which does not have this disadvantage.

SUMMARY AND CONCLUSIONS

1. Clinical experience with the use of an improved fat emulsion for intravenous administration in 151 subjects and 362 infusions is compared to prior results with an emulsion of similar composition in 110 subjects and 293 infusions. The emulsions differed only in the purification of the soybean phosphatide emulsifier.

2. The over-all reaction rate indicates that 51 per cent of the subjects had one type of reaction or the other with the old emulsion as contrasted to 33 per cent with the new one. However, fever accounts for 88 per cent of the total number of reactions with the new emulsion, as compared to 57 per cent with the old. Thus, reactions other than fever have been drastically reduced. This is confirmed by the fact that only 3 out of 362 infusions could not be completed with the improved emulsion as compared to 23 out of 293 infusions with the former preparation. Not a single instance of severe or frightening reaction has been encountered with the improved emulsion.

3. The data accumulated on the improved emulsion constitute, in our opinion, the best results thus far reported with any fat emulsion in a large series of ill patients and indicate that this preparation is a practical, usable material.

4. The instability of the emulsion to extreme environmental conditions, electrolyte solutions, and amino acid solutions would seem to point up the importance of continuing efforts to improve the product.

The author wishes to express his appreciation to Capt. M. R. Allen, Sgt. Hugh Gray, and Sgt. Chelsia Goodman for their excellent and devoted nursing and clerical help, and without whose services the above study could not have been completed. He would also like to thank Dr. Henry Swan, Professor of Surgery, University of Colorado School of Medicine, and his house staff for their kind cooperation in furnishing us with most of the patient material.

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STUDIES OF THE PHENOMENON OF THE CLEARING OF INFUSED FAT EMULSIONS FROM HUMAN BLOOD AND ITS RELATIONSHIP TO THE FEBRILE REACTION

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IN THE preceding paper, clinical results with an improved fat emulsion for intravenous use in humans were reported. The incidence of adverse side reactions as compared with a former preparation was markedly reduced, with the prominent exception of fever which occurs in 29 per cent of the patients. The improvement in the reaction rate is ascribed to the removal of "impurities" in the soybean phosphatide utilized as the emulsifier in the recent emulsion. The fact that the febrile reaction was not affected by the purification of the phosphatide suggested that fever may result from some physiologic mechanism. The purpose of this report is to present data which are directly related to this problem and support the concept of "physiologic" fever.

The febrile response is a well-recognized complication of the administration of intravenous fat emulsions to humans. Many investigators have searched for its causation, but none have satisfactorily explained it. Such obvious possibilities as pyrogens, particle size, unesterified fatty acid content, and peroxide content have been eliminated as etiologic agents.[1] In vivo hemolysis has been incriminated in the past, as well as more recently by Creditor.[2] The latter author demonstrated marked increases in mechanical fragility of the red cell in the presence of a fat emulsion and demonstrated hemoglobinemia following an infusion of fat. However, the presence of hemoglobin in lipemic plasma, considering the marked increase in mechanical fragility of the red cells, may only indicate trauma coincident with venipuncture and therefore not reflect in vivo hemolysis. That this is true is suggested by studies from our laboratory, in which the amount of bilirubin in bile collected from indwelling T-tubes in the common duct was measured before and after an infusion of fat.[3] No increase in total amount of bilirubin was found following the infusion. Furthermore, recent studies by Levenson and Upjohn[4] on red cell survival utilizing the Cr^{51} technique failed to show any effect of multiple fat infusions on the survival curve.

Our laboratory has been interested for some time in the problems relating to the clearing of fat from the blood and has performed some studies in normal volunteers on the clearance of intravenous fat emulsions. It seemed

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The opinions expressed in this paper are those of the author and do not necessarily represent those of any governmental agency.
Received for publication Jan. 28, 1957.
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pertinent to extend these observations to a large series of sick persons. Johnson, Freeman, and Meyer,[5] using an emulsion containing 10 per cent olive oil, demonstrated a difference in the clearing rates in a small series of patients with liver, thyroid, or renal disease. It soon became obvious from our studies that there was a great individual variation among patients in their ability to remove the injected fat from the circulatory system. There also appeared to be a direct correlation between the rapidity of clearing and temperature elevation. These and other related observations on clearing are the basis on this report.

MATERIALS AND METHODS

A heterogenous group of hospitalized patients at the Colorado General, Denver General, and Veterans Administration Hospitals in Denver, Colorado, were studied. Eighteen normal volunteers were studied also and are included in the series. No attempt has been made to select patients either for severity or specificity of disease with the exception of a group of patients with cirrhosis of the liver.

The composition of the "new" emulsion and method of administration were outlined in the preceding paper. Since we were interested in clearing rates, the flow and total time of infusion were carefully controlled and kept as constant as possible. The average time of administration was 216 minutes. The bottle of emulsion contained 90 Gm. of fat; therefore, the flow was approximately 25 Gm. per hour in each subject. Each infusion was begun in the morning before a low-fat breakfast. Lunch was also a low-fat meal and was given after the infusion was completed.

To estimate clearing rate, the following test was devised. The optical density of the subject's serum was measured before, at the end, and eight hours after the beginning of the fat infusion. The optical density (O.D.) was measured with a Coleman Jr. Spectrophotometer at 700 mμ, using water as the blank. The bloods were kept at 40° F. temperature during the test day and read the morning following the day of infusion.

Body temperature of the recipient was measured hourly for a period of twelve hours. Rectal temperatures were recorded whenever possible. A febrile reaction has been defined as a rise in temperature of at least 2° F. above 99.6° F. rectally, or 2° F. over the control temperature, if that figure is above "normal."

Soon after the start of the study, it became apparent that there was a great variability among patients in the rapidity with which their blood cleared of the infused fat. At first, arbitrary figures for the optical density at the various sampling times were set in an effort to categorize the patients according to fast, slow, or normal clearing. Thus, those patients whose serum had an optical density above 1.0 at the end of the infusion and had returned to preinfusion levels by 8 hours were considered normal clearers. If the plasma remained lipemic after 8 hours, the subject was said to be a slow clearer; if the optical density was below 1.0 at the end of the infusion, then he was said to be a fast clearer. These arbitrary criteria were based on previous experience with normal volunteers. However, it became obvious that delineation into fast, slow, or normal clearing patterns was not practical, since some of our volunteer subjects did not fit the "normal" pattern. It seems preferable, therefore, to refer to actual optical density readings rather than arbitrary limits, reminding the readers that an optical density figure above 0.3-0.4 represents grossly lipemic serum.

Optical density is a simple, convenient means of measuring lipemia. Other techniques, such as chylomicron counts or nephelometry, have well-recognized limitations and, in the case of the former, are time consuming as well as fraught with error.[6] Optical density measures transmission of light across a given path, so that any particles present which reflect or diverge the path of light will affect the optical density. Thus, the latter is proportional to the concentration of particles; in our case, concentration of fat particles. Since all of the fat administered in the emulsion is in the form of chylomicrons,

the clearance of these fat particles, therefore, can be followed with optical density measurements. This assumes, of course, that most of the fat leaves the blood stream as chylomicrons rather than first becoming solubilized to lipoprotein. The correlation between optical density and total fatty acids following an infusion of fat emulsion is shown in Fig. 1. The sigmoid curve reflects the inefficiency of the Coleman spectrophotometer at the upper level and the individual fasting variations in soluble fat at the lower level. However, in the ranges in which we are most interested, that is, O.D. from 0.3 to 1.5, there is a moderately good straight line relation between total fatty acids and optical density. Interfering pigments, such as bilirubin and hemoglobin, will affect the readings to a small, insignificant degree.

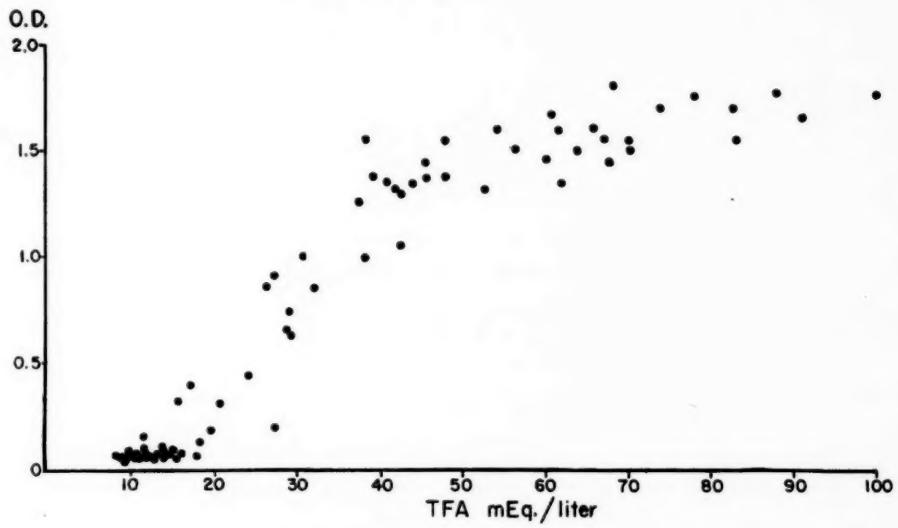

Fig. 1.—The relationship between the optical density of the whole serum and the total fatty acids of the same whole serum.

RESULTS

In all, 225 clearing studies have been performed on 120 subjects infused with the "new" emulsion. The large amount of data accumulated does not permit detailed presentation of individual records or results. An insight into some of this information can be gained from the accompanying graphs. Several means are available to assess the rate of clearing in an individual and make comparisons among individuals. Since a constant amount of fat is infused at a constant rate and the clearing process is a dynamic one, the optical density readings at comparable times can be used. Thus, in Fig. 2 the optical density of serum at 8 hours is plotted against the highest rectal temperature reading in the 12-hour period of the experiment for 95 subjects receiving their first infusion. With two exceptions, significant fever did not occur in those subjects whose serum was lipemic (O.D. $>$ 0.3) after 8 hours. Conversely, those patients who develop a febrile reaction to the intravenous fat have essentially clear serum at 8 hours, although fever does not invariably result from rapid clearing. The same relationship is obtained when the optical density at the end of infusion is plotted against temperature as in Fig. 3. In both instances the correlation is highly significant ($P < 0.01$). Since

clearing is a dynamic process, the change in optical density during infusion and the per cent decline in optical density after infusion when compared with temperature rise, likewise show a highly significant correlation ($P < 0.01$).

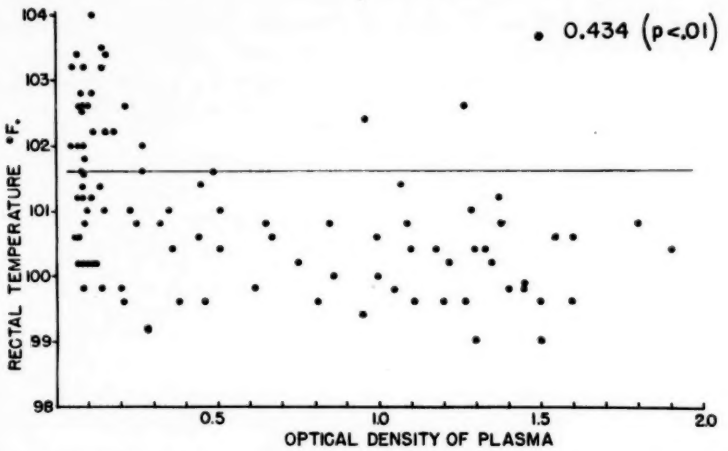

Fig. 2.—Relation between the optical density of the plasma 8 hours after the start of an infusion of 600 ml. of Lipomul and the highest rectal temperature in the 12-hour post-infusion period in 95 subjects receiving their first infusion.

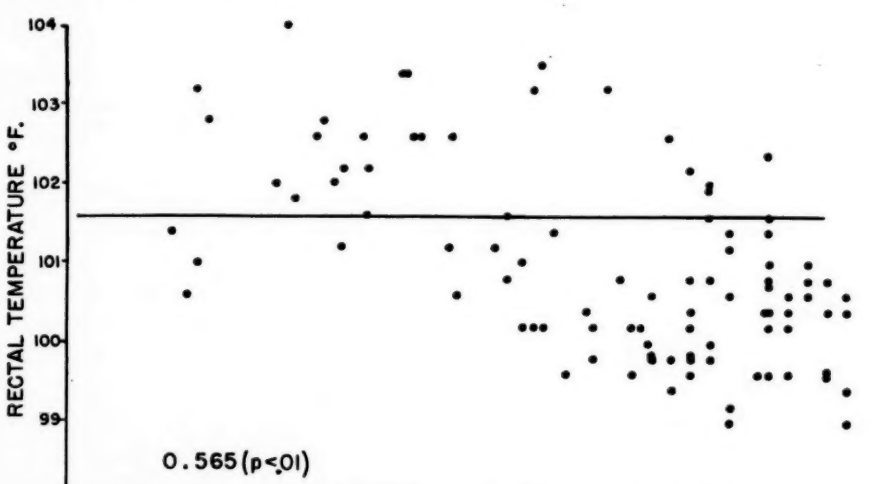

Fig. 3.—Relationship between the optical density of the plasma immediately after the end of an infusion of 600 ml. of Lipomul and the highest rectal temperature in the 12-hour postinfusion period in 95 subjects receiving their first infusion.

When the data are analyzed for 168 infusions, which represent repeat infusions in some subjects, the correlation between the optical density at the end of infusion and at 8 hours and the highest rectal temperature again is significant at the 1 per cent level of probability. However, if one analyzes the results for the 73 repeat infusions alone, significance is not attained (Fig. 4).

Some interesting observations have been made concerning the clearing pattern on repeat infusions in the same individual. Thirty such studies have been made, of which a representative number have been depicted in Fig. 5.

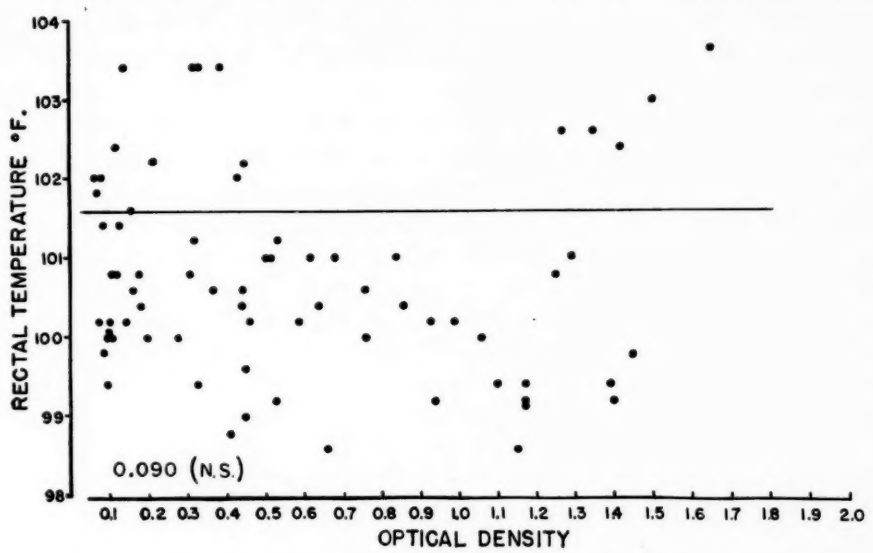

Fig. 4.—Relationship between the optical density of the plasma 8 hours after the start of an infusion of 600 ml. of Lipomul and the highest rectal temperature in the 12-hour postinfusion period of 73 repeated infusions.

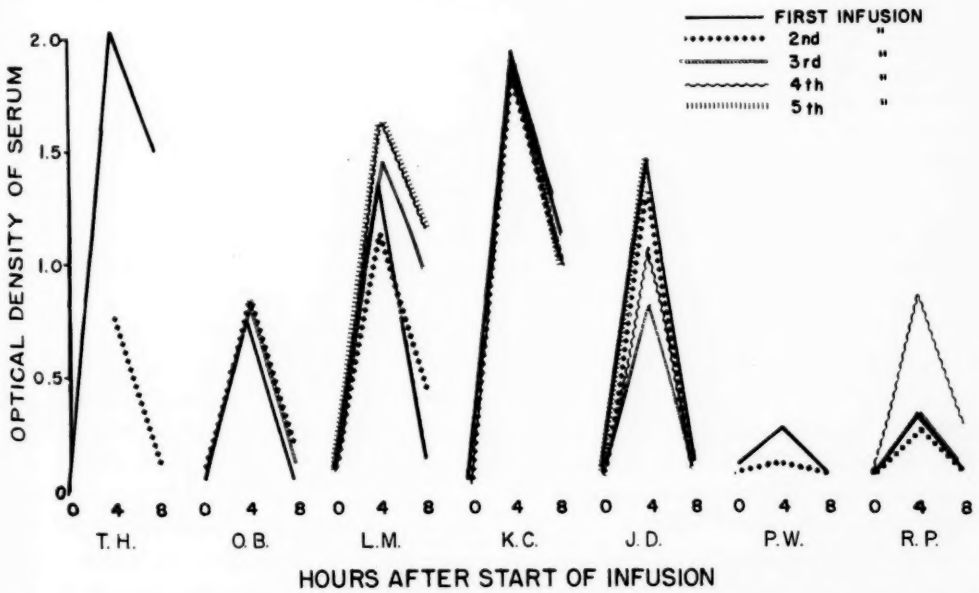

Fig. 5.—Representative examples of individual patients' clearing test following 600 ml. of Lipomul on successive days.

It is clear from this sample that the previously mentioned variation in first infusions is also present in successive ones. There is no regular sequence of clearing in each individual. The normal individuals, as well as patients

with cirrhosis, tend to clear slower with successive infusions up to a point. However, there is so much individual variation within the group that statistical analysis of the data was worthless. In patients with cirrhosis receiving a total of 20 infusions, after the first 4 or 5 infusions there was no clear-cut pattern, so that the clearing on infusion number 20 was not necessarily the slowest of the entire study.

With reference to Fig. 5, Patient T. H. represents an interesting phenomenon. The two infusions were carried out within 48 hours of one another. The patient had undergone a subtotal gastrectomy for gastric ulcer and had the additional diagnosis of ulcerative colitis. The general condition of the patient on the day of the first infusion was good. Although great weight loss had occurred, oral feedings had just been begun. The infusion of fat was well tolerated and cleared slowly from the blood as the chart indicates. However, the next day vomiting and abdominal cramps recurred, oral feeding was discontinued and gastric suction reinstituted. The following day another bottle of fat was administered. As is evident, clearing occurred very rapidly. The tremendous difference in the clearing pattern in this individual under these circumstances suggested that rapidity of clearing may depend, to some extent, upon the needs of the body for energy.

Patient P. W. is an example of the magnitude of the body's ability to clear the infused fat. He was an 83-year-old white man suffering from severe generalized arteriosclerosis which culminated in a thrombosis of the left femoral artery requiring ultimately an amputation above the knee of the involved leg. His general condition was quite poor; tracheotomy had been performed and parenteral alimentation was necessary. During two successive infusions, separated by one day, the fat was cleared so rapidly that clear serum was present at the end of each infusion. As would be expected, temperature elevations to 101.4° F. and 101.8° F., respectively, occurred. This is an amazing demonstration of the capacity of the body's mechanism for removing fat and points up the need for further studies into the nature of this mechanism.

DISCUSSION

The results of the clearing studies indicate a direct correlation between the rapidity of removal of the infused fat and the temperature elevation subsequent to the administration of the emulsion. Those persons in whom the fat is removed slowly from the blood do not experience a febrile reaction. The two exceptions to this observation both had cirrhosis of the liver and were febrile on the days before and after the fat infusion. It is possible, therefore, that the fat had no relation to the febrile response in these two instances. The interpretation of these observations would support the hypothesis that the fat, which is rapidly removed from the blood stream, is likewise rapidly oxidized, and the endogenous heat liberated thereby cannot be dissipated rapidly enough and fever results. The studies of Shafiroff and colleagues[7, 8] on the early utilization of intravenous fat and oxygen consumption studies on humans receiving emulsified fat would support such a hypothesis.

An alternative explanation might be that the oil contains a pyrogenic substance which is liberated in larger quantities in a given period of time during the process of fast clearing. However, previous studies by Waddell and associates,[9] with an emulsion containing a synthetic oil, triolein, showed no significant decrease in the thermogenic response as compared to emulsions composed of natural fats. Our own experience with an emulsion containing cottonseed oil which had been specifically "cleaned up" and incorporated into the regular formula likewise showed no decreased thermogenicity in humans. Lambert, Miller, and Frost[10] studied thermogenicity of several types of emulsions containing either synthetic or natural fats. The only nonthermogenic emulsion they could prepare was one in which triton was incorporated, which specifically slows down the clearing of the emulsion. It would appear, therefore, that impurities in the oil are an unlikely cause of the febrile response, and a more physiologic mechanism as outlined above seems more reasonable.

The fact that individuals may clear their blood rapidly and not develop fever is difficult to explain. Variation in susceptibility to febrile stimuli in the general population is well recognized and thought to be due to vagaries of the thermoregulatory center in the midbrain. In our study, it was a clinical impression that the more severely ill patients tended to develop fever more often than the less severely ill. There are no objective criteria on which this impression can be based, since severity of illness cannot be objectively delineated. However, if one accepts the clinical impression, then the primary illness of the patient may "sensitize" the thermoregulatory mechanism in such a manner that the more severely ill patient will develop fever consequent to fast clearing, whereas the normal or less ill subject will not.

If the febrile response is dependent upon the rate at which the infused fat leaves the blood, then, regardless of the number of infusions or methods of administration, this relationship should always hold. The data on the 73 repeat injections appear to refute the interpretation. However, all of the discordant observations of slow clearing and high fever in this group are composed of results of repeat infusions in the two subjects with cirrhosis mentioned above. We did not feel justified in removing these subjects from our series, but we do feel that the apparent discrepancies between first infusions and repeat infusions may be explicable on this basis. Another explanation exists, of course, that unknown factors other than rate of clearing influence the febrile reaction in certain instances, particularly in subjects receiving multiple infusions. There is no information at present either to confirm or to deny this possibility.

The two specific case reports cited in the section on "Results," as well as the clinical observation that the sicker patients clear faster than the more healthy subjects, suggest that the nutritive status of the recipient may affect the rate of clearing.

The role of "clearing factor enzyme," first described by Hahn,[11] in the physiologic clearing process is certainly unclear. This hydrolytic enzyme has

been found in human blood in appreciable amounts only as a result of the injection of heparin or a like substance. Cleland and Iacono[12] in our laboratory have measured the amount of clearing factor activity present in the blood of some of the subjects following an infusion of fat, utilizing a method which measures glycerol production in vitro over a 4-hour period. None of the subjects was heparinized. They have found an insignificant amount of enzyme present which could account for the hydrolysis of approximately 5 per cent of the fat which is cleared. Thus, it would appear that clearing factor enzyme, better referred to as lipoprotein lipase, plays a very insignificant role in the actual clearing of the blood.

The role of the reticuloendothelial system in the clearing process has been extensively studied by others.[13] Histologic scrutiny of tissues of animals immediately following the infusion of a fat emulsion has shown fat globules in macrophages of the spleen and Kupffer cells of the liver.[14] However, the experiments by Waddell and co-workers[13] in rats showed very clearly that blockage of the reticuloendothelial system with India ink or trypan blue did not affect the clearing of an injection of fat emulsion from the blood, but that evisceration and, in particular, hepatectomy, grossly slowed the clearing.[15]

The studies herein reported do not shed much light upon the role of the liver in the complex clearing mechanism. Of the 11 cirrhotic patients in this series, all but one were slow clearers. However, so were most of the normal volunteers. This is in distinct contrast to the studies of Johnson and associates,[5] in which the cirrhotics cleared faster than normal. Unfortunately, none of our patients were suffering from acute liver disease or intoxication, so that the observations by Scott and Vars[16] that chloroform intoxication of the liver increased the clearing of fat could not be verified.

Recently, interest has developed in the effects of aging and, in particular, atherosclerosis, on the disappearance rate of lipemia produced by an oral fat meal. Necheles and co-workers[17, 18] reported that aged individuals clear their blood of ingested fat more slowly than younger persons, although White, Ralston, and Carne[19] could not corroborate this effect but found instead a correlation between bed rest and slow clearing. Moreton[20] first called attention to the fact that patients with atherosclerosis clear more slowly than those without this affliction. These observations have been confirmed by Pomeranze, Beinfield, and Chessin[21] in the obese and atherosclerotic patient, and by Schwartz, Woldow, and Dunsmore[22] in patients with myocardial infarction. Utilizing I^{131}-labeled neutral fat by mouth, Thannhauser and Stanley[23] demonstrated slow clearing in patients with idiopathic hyperlipemia as compared to normal subjects. All of these observations are complicated by the process of fat absorption, so that elevated or prolonged blood lipid level after a fat meal may not, of necessity, reflect the activity of the clearing mechanism, but rather an absorptive phenomenon.

Our study was not designed to investigate any specific disease. However, because of the interest in the possible etiologic relationships between lipemia and various disease states, a review of our case material is presented

in Table I with these thoughts in mind. Because of the arbitrary categorization into slow, intermediate, and fast clearing patterns and the small number of subjects in certain areas of interest, it is only possible to derive trends or suggestions from these data. However, it is of interest to note that as weight increases, slowness of clearing likewise becomes more common. There is no apparent correlation between the presence or absence of diastolic hypertension and the pattern of clearing. The converse of the results obtained with an oral fat load in various age groups was found in our studies, wherein increased clearing was associated with increasing age. The types of clearing

TABLE I. THE RELATION OF VARIOUS PHYSIOLOGIC PARAMETERS AND DISEASE STATES AND THE PATTERN OF CLEARING OF THE BLOOD FOLLOWING AN INFUSION OF A FAT EMULSION

		CLEARING PATTERN					
	TOTAL	INTERMEDIATE*		SLOW†		FAST‡	
Weight	NO.	NUMBER	(%)	NUMBER	(%)	NUMBER	(%)
Less than 100 pounds	8	6	(75)	2	(25)	0	(0)
100-150 pounds	76	27	(35)	28	(36)	21	(29)
150-200 pounds	14	6	(43)	6	(43)	2	(14)
Over 200 pounds	1	0	(0)	1	(100)	0	(0)
Blood Pressure							
Diastolic above 90 mm. Hg	28	16	(57)	8	(28)	4	(15)
Diastolic below 90 mm. Hg	77	28	(36)	29	(37)	20	(27)
Age							
20-40 years	21	7	(33)	12	(57)	2	(11)
40-60 years	39	11	(28)	20	(51)	8	(21)
60-80 years	54	28	(52)	11	(20)	15	(28)
Clinical Diagnosis							
Hyperlipemia	1	0	(0)	1	(100)	0	(0)
Diabetes mellitus	2	1	(50)	1	(50)	0	(0)
Coronary occlusion	5	1	(20)	4	(80)	0	(0)
Stroke	7	3	(43)	4	(57)	0	(0)
Arteriosclerotic heart disease	9	4	(44)	1	(12)	4	(44)
Generalized arteriosclerosis	17	8	(47)	4	(23)	5	(30)
Family history of any of the above	21	6	(28)	11	(52)	4	(20)
Cirrhosis of liver	11	0	(0)	10	(91)	1	(9)
Normal volunteers	18	6	(33)	12	(66)	0	(0)

*Intermediate = O.D. above 1.0 at end of infusion, below 0.3 after 8 hours.
†Slow = O.D. above 1.0 at end of infusion, above 0.3 after 8 hours.
‡Fast = O.D. below 1.0 at end of infusion, below 0.3 after 8 hours.

patterns observed in the various disease entities are confusing and probably reflect the complex nature of the clearing phenomena. These patients were suffering from either complications of their primary illness or some other unrelated affliction which undoubtedly affects the clearing pattern and leads to the vagaries noted. One would like to study the various illnesses in an uncomplicated form, and, even then, the inborn variation in the physiologic phenomena of clearing may cloud the issue.

SUMMARY AND CONCLUSIONS

1. A clearing test has been devised which measures the rapidity at which the blood is cleared of infused fat. It has been applied to the study of 120 subjects receiving 225 infusions of an emulsified fat preparation.

2. Twenty-nine per cent of the subjects receiving the fat developed a significant elevation of body temperature.

3. There is a highly significant correlation between the rapidity of clearing and the febrile response.

4. These results are interpreted as evidence that the febrile response to intravenous fat therapy may be due to rapid oxidation of the fat with resultant endogenous heat production and should be considered a physiologic reaction in susceptible individuals. The nature of the fever is such that it is not considered a contraindication to the use of fat emulsion for parenteral alimentation.

5. The possible reasons for the great variability in clearing patterns between individuals, as well as between successive infusions in the same individual, are discussed. Until the basic physiologic mechanism regulating fat transport in the blood is better understood, the vagaries will remain obscure.

6. Lines of future investigation are cited, and it is hoped that the fat emulsion will prove a useful tool in future studies of fat metabolism.

The author wishes to thank Mrs. Lucille Palm Wendel for valuable technical aid and Capt. M. R. Allen, Sgt. Hugh Gray, and Sgt. Chelsia Goodman for expert nursing and clerical help.

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THE EFFECTIVENESS OF MEPROBAMATE AND LOBELINE AS SMOKING DETERRENTS

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EVER since its introduction, the use of tobacco has been criticized by various groups for varied reasons. The pressure on a smoker to quit has been increased of late, chiefly because of reports linking tobacco use with a higher incidence of bronchiogenic carcinoma. There is also some concern regarding the role of tobacco in peptic ulcer, coronary artery disease, peripheral vascular disease, and other bronchopulmonary disease. Disregarding for the moment the part that tobacco plays in these diseases, let us consider what can be done to help the patient who wants to stop smoking or who has been advised by his physician to quit.

In 1936, Dorsey reported encouraging results with the use of lobeline.[1] Wright and Littauer later reported that the side effects of lobeline were such as to preclude its general use.[2] Rapp and Olen recently published a study which indicated that they had circumvented the side effects by combining lobeline with an antacid mixture; they stated that the preparation was very effective in reducing the desire to smoke.[3] Another preparation for the relief of withdrawal effects has been reported; it contains $\frac{1}{20}$ grain benzocaine flavored with saccharin, extract of liquorice, powdered ginger, and oils of anise, wintergreen, peppermint, coriander, and cloves.[4] However, it is our opinion that this study was not sufficiently controlled to warrant prescribing this aromatic preparation. Other methods have been proposed from time to time, but none have been successful.

Johnston states that the only way to stop smoking is by not smoking again.[5] This Spartan dictate is often ignored by patients, who feel that they need something more to help them through the withdrawal period. Johnston feels that there are two components involved in the desire to continue smoking; the first is a pharmacologic craving which is of high intensity but of short duration, reaching a peak 24 to 36 hours after stopping, while less intense psychologic urges may persist for years. Obviously, a drug which could help prevent the patient from starting to smoke again in the first few weeks would be very useful.

The popularity of the ataraxic drugs seemed to offer a new attack on this problem. Since most cigarette smokers state that a smoke "relaxes" them, and

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The drugs used in this study were furnished by Wallace Laboratories (Miltown) and Compana Sales Corporation (Bantron). A grant from Wallace Laboratories was used to defray expenses.

Received for publication Feb. 4, 1957.

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since the ataraxics also "relax" the patient, we felt that the effect of a tranquilizer might logically be expected to reduce the need for cigarettes. We also wished to check the efficacy of the lobeline-antacid mixture.

PROCEDURE: PART I

Thirty-three young, adult medical students were used, each of whom stated that he was in good health. These subjects were divided into 4 groups; the drug schedule was so arranged that the order of drugs was different for each group, but each subject still received all 3 preparations. The tests were run in 3-week cycles; the first control week was a determination of the number of cigarettes smoked normally for a 7-day period, while the second week was a determination of the cigarettes smoked while taking one of the drugs. There followed a third recovery week wherein the subject took neither a preparation nor kept a record of the cigarettes smoked (Table I).

TABLE I. SCHEDULE OF DRUG ADMINISTRATION IN PART I

	WEEK OF TEST							
SUBJECTS	1	2	3	4	5	6	7	8
1- 9	Control	Placebo	Recovery	Control	Bantron	Recovery	Control	Miltown
10-18	Control	Bantron	Recovery	Control	Miltown	Recovery	Control	Placebo
19-27	Control	Placebo	Recovery	Control	Miltown	Recovery	Control	Bantron
28-33	Control	Miltown	Recovery	Control	Bantron	Recovery	Control	Placebo

The drugs tested were meprobamate, and a commercial preparation known as Bantron, which was stated to be identical with that used by Rapp and Olen. A sugar placebo was included, and all drugs were powdered and placed in identical No. 0 pink capsules to be taken 3 times daily. A capsule contained either (1) 400 mg. meprobamate, (2) the equivalent of 1 tablet of Bantron, or (3) sugar. The subjects were aware of the fact that they might be given a placebo, but they were not told which preparation they were receiving at any given time. They were instructed to make no attempt to influence their smoking level, but merely to have a cigarette when they felt they needed one. It was felt that this procedure would measure any depressant effect on smoking, and would tend to eliminate the factor of "will power."

The subjects were told that we had reason to believe that the drugs they were being given would probably reduce their cigarette consumption. The methods used for evaluation were those of the double-blind technique.

RESULTS: PART I

The thirty-three subjects had smoked an average of 8½ years, and at the time of the test were smoking around 16 cigarettes per day. The results were

TABLE II. AVERAGE NUMBER OF CIGARETTES SMOKED PER DAY PER SUBJECT FOR 33 SUBJECTS

	DAY OF TEST						
	1	2	3	4	5	6	7
Control	16.8	17.9	18.3	17.9	17.6	16.1	15.8
Bantron	16.7	17.2	16.0	17.4	17.4	15.4	16.1
Control	17.8	16.9	18.1	18.4	19.2	16.0	17.3
Placebo	16.9	16.2	16.9	17.2	16.6	15.9	15.0
Control	17.5	17.0	17.4	17.4	17.9	17.1	16.2
Miltown	16.1	17.0	17.4	17.9	17.1	15.5	15.6

calculated for the group as a whole in terms of the average number of cigarettes per day per subject. In analysis, it was observed that neither meprobamate nor the commercial lobeline preparation was significantly more effective in reducing

the smoking levels than was the sugar placebo (Table II). The group was then subdivided into subgroups of those smoking between 5 and 14.9 per day, between 15 and 24.9 per day, and over 25 per day and the data re-evaluated. The results of this analysis did not differ significantly from that of the group as a whole. We are at a loss to explain the great discrepancy between our results and those of Rapp and Olen.[3]

PROCEDURE: PART II

It could be argued that we were not measuring the effect of the drugs to replace cigarettes by the above procedure. Hence, we obtained volunteer subjects who expressed a strong desire to stop smoking. These subjects were arbitrarily divided into two groups, one of which was given capsules containing 400 mg. meprobamate, and the other given indistinguishable placebo preparations. The subjects were instructed to take a capsule whenever they felt the withdrawal symptoms start to appear, but not to take over 8 capsules per day. They were further told that the drug would be effective in reducing the desire for a cigarette. When the tests were completed, the groups were as follows:

Miltown Group: 6 men and 3 women, smoking for an average of 9.3 years and smoking an average of 17.5 cigarettes per day prior to the test

Placebo Group: 7 men, smoking for an average of 8.4 years and smoking an average of 21.6 cigarettes per day just preceding the test

RESULTS: PART II

No significant difference was noted between the two groups, either by success in quitting or by their impression of the effectiveness of the drug. It was of especial interest that only one of the subjects given the placebo felt that this had not helped him (Table III).

TABLE III. COMPOSITION OF TEST GROUPS AND RESULTS (PART II)

	MEN	WOMEN	AVERAGE YEARS SMOKING	AVERAGE DAILY CIGARETTE USE	SUBJECTS CEASED SMOKING	SUBJECTS FELT DRUG WAS OF VALUE
Miltown	6	3	9.3	17.5	3	8
Placebo	7	0	8.4	21.6	3	6

DISCUSSION

In spite of the favorable reports in the literature on the effectiveness of lobeline, we were unable to demonstrate that the preparation used has any effect on cigarette consumption. Similarly, although the use of a tranquilizing drug would appear indicated, the one used in this study, given in the manner described, had no effect. Since the subjects were told to take a capsule whenever they first noticed the symptoms of withdrawal start to appear, we feel that the time necessary for the drug to be released from the capsule, absorbed, and exert its effect may have been excessive. In other words, the symptoms may have become intolerable before the drug took effect. A faster acting drug, or perhaps a high "prophylactic" daily dose, might be more effective.

The present treatment of tobacco withdrawal effects would seem to be psychological; we know of no currently used pharmacologic agent which has a clearly effective action. A procedure to be used when dealing with a patient who

must or desires to stop smoking might be as follows: first, make sure that he really wants to quit; if this motivation is lacking, no success can be expected. Second, reinforce that desire by telling him of studies incriminating tobacco as a pathogenic agent. And finally, support him in his endeavor by other psychologic measures, including the use of sugar capsules if deemed necessary.

SUMMARY

In a study of the smoking habits of thirty-three medical students, no effect was noticed upon administration of meprobamate or a commercial buffered lobeline preparation.

In a study involving 15 medical students who stated they wished to stop smoking, no objective or subjective difference was noted between the group receiving meprobamate and those receiving a placebo. However, 6 out of 7 subjects felt that the placebo had helped them to resist the desire to smoke.

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DETERMINATION OF AROMATIC ACIDS AND PHENOLS IN THE URINE OF WHITE AND NEGRO MALE ADULTS

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THE possible relationship of tyrosine and phenylalanine metabolism to phenylacidurias in tyrosinosis, mental disease, vitamin C and folic acid deficiencies, etc., and also of the enzymatic oxidation of tyrosine by tyrosinase and dopa-oxidase to aromatic acids and the melanin pigments still remains obscure.

As a possible tool in studying these conditions, microbiologic methods were developed for the assay of phenyllactic, *p*-hydroxyphenyllactic, and indole-3-lactic acids in urine. In addition, ether extracts of these urines were analyzed for total volatile phenols and aromatic hydroxy acids by previously described methods[1, 2] which utilize the Folin-Ciocalteau reagent. Perhaps the Negro, due to the presence of large amounts of dopa-oxidase in the skin, might excrete increased amounts of phenylaciduric or phenolic material. Preliminary experiments had encouraged us in this belief.[3] Also, recent work indicates that Chinese, for example, excrete significantly greater quantities of amino acids in urine than Caucasoids and that diet is not a principal factor in this difference.[4]

MATERIAL AND METHODS

Twenty-four-hour specimens of urine were collected for 2 consecutive days from apparently healthy Negro and white male adults. Forty milliliter aliquots of each urine were acidified with 10 N sulfuric acid to pH O and refluxed for one hour. The hydrolysates were adjusted to 200 ml. at pH 10 with dilute sodium hydroxide and then extracted continuously with redistilled (peroxide-free) ether for 24 hours.[1, 5] Each extract was analyzed for the volatile phenol fraction with the Folin-Ciocalteau reagent as previously described.[2] The residue in each extractor was acidified with 2 ml. of 10 N sulfuric acid and extraction continued for 24 hours. This extract contained the aromatic acid fraction. The ether was volatilized and the aqueous residue neutralized to pH 6.9 and diluted to 25 ml. The total aromatic hydroxy acid content was also determined with the Folin-Ciocalteau reagent.[2] Then, individually, phenyllactic, *p*-hydroxyphenyllactic, and indole-3-lactic acids were determined with *Lactobacillus casei* (ATCC No. 7469) as described below.

Our microbiologic assay procedures, media, cultures, inocula, etc., have been described elsewhere.[1, 6] The basal media were premixed crystalline preparations of nutrients.† Each preparation was nutritionally complete for *L. casei* except for the specific amino acid whose hydroxy analog was being assayed. A weighed quantity of each medium was freshly dissolved in distilled water and adjusted to pH 6.9.

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Supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

Received for publication Feb. 4, 1957.

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†Manufactured by the H. M. Chemical Company, Santa Monica, California.

Pyrex tubes (18 × 150 mm.) were immersed in chromic acid cleaning solution, washed with water 10 times, autoclaved with distilled water, and then dried. A 1 ml. quantity of the appropriate basal medium, depending upon the amino acid analog being assayed, was added to each tube. A 0.5, 1.0, or 2.0 ml. aliquot of a sample was added and the contents of each tube were made up to 5 ml. with distilled water. Each sample was assayed in replicate.

The tubes were covered with aluminum caps, autoclaved at 15 pounds pressure for 15 minutes, cooled, inoculated, and then incubated for 72 hours at 34° C. Since urine extracts are frequently colored, the acidity produced by the organism was titrated electrometrically.[7] With each urine assay, standard growth curves were developed simultaneously with phenyllactic acid, *p*-hydroxyphenyllactic acid, and L-tryptophane (indole-3-lactic acid was not available). If any keto analogs of these amino acids were present in the urines, they would be destroyed by the drastic acid hydrolysis.[8]

When 200 to 20,000 mcg. of each hydroxy analog was added to urine and hydrolyzed at pH O, extracted, and assayed as described above, recovery values ranged from 86 to 102 per cent. The addition of 2 mg. of Tween 40 per tube (data omitted), to eliminate the inhibitory action of any oleic acid present in the urine extracts, did not change the results of the assay.

Previous work from this laboratory demonstrated that significant changes in the concentration of urinary phenolic bodies were not observed between human subjects on high and low protein diets. This effect was observed only when protein was rigidly eliminated from the diet.[9] Therefore no check on food intake was made except by a total urine nitrogen determination. Each subject was warned repeatedly to avoid medicines or drugs.

RESULTS

The results of the analysis of two consecutive 24-hour urine samples from 10 individuals are reported in Table I. It is obvious that the values for the various urinary constituents, especially the microbiologically determined aromatic acids, vary widely from subject to subject although the two consecutive specimens in each case show some degree of uniformity. These variations, however, are not surprising in view of the work of Williams and his colleagues who concluded that every individual possesses a distinctive metabolic personality or pattern and that values for various urinary constituents obtained from different individuals often differ by as much as several hundred per cent.[10]

p-Hydroxyphenyllactic acid was present in small amounts, ranging from 318 to 1,475 mcg., average 860 mcg., for the urines from the whites and 166 to 1,465 mcg., average 699 mcg., for those from the Negroes. Indole-3-lactic acid was also present in small amounts, ranging from 108 to 486 mcg., average 341 mcg., and 193 to 821 mcg., average 500 mcg. for the same urines. The amount of phenyllactic acid ranged from insignificant traces to quantities not exceeding 406 mcg. per 24-hour samples.

The total aromatic hydroxy acids determined with the Folin-Ciocalteau reagent on ether extracts and calculated as *p*-hydroxyphenylacetic acid ranged from 155 to 450 mg., average 253 mg., and 174 to 258 mg., average 219 mg. for the two groups of urine samples. Here relative variations between urines are somewhat smaller. Thus, the 3 amino acid analogs determined microbiologically constitute less than one per cent of the total aromatic hydroxy acid fraction determined chemically and calculated as *p*-hydroxyphenylacetic acid.

It appears obvious, however, from the good recovery values obtained for the microbiologically determined aromatic acids, that the relatively larger quantities of other aromatic acids in the urines did not interfere with the utilization of the amino acid analogs by *L. casei*. Previous work[3] from this laboratory showed that the various aromatic acids normally present in urine are relatively poor inhibitors of the metabolic conversion of phenyllactic acid to phenylalanine by *L. casei*. In this respect, however, mandelic acid proved to be a potent inhibitor.

TABLE I. AROMATIC ACIDS AND PHENOLS IN URINE FROM WHITE AND NEGRO MALE ADULTS*

SUBJECT	RACE	VOLATILE PHENOLS (MG.)	AROMATIC HYDROXY ACIDS (MG.)	PHENYL-LACTIC ACID (MCG.)	*p*-HYDROXY-PHENYL-LACTIC ACID (MCG.)	INDOLE-3-LACTIC ACID (MCG.)	TOTAL NITROGEN (GM.)
K.	White	117	228	78	469	254	14.1
K.	White	117	256	I†	516	258	14.9
V.	White	91	182	69	318	486	11.0
V.	White	125	165	I	354	267	11.6
H.	White	224	437	I	1,131	108	15.7
H.	White	302	450	I	1,151	200	15.9
W.	White	172	255	264	1,475	395	16.2
W.	White	127	204	I	1,400	467	11.4
S.	White	119	155	406	922	601	12.0
S.	White	136	196	117	860	371	12.0
Max.		302	450	406	1,475	486	15.9
Min.		91	155	I	318	108	11.4
Ave.		153	253	--	860	341	13.5
M.	Negro	147	226	156	547	332	11.4
M.	Negro	166	209	234	719	352	13.6
P.	Negro	163	196	I	166	193	10.3
P.	Negro	146	174	I	183	414	9.1
Q. J. R.	Negro	161	250	116	679	475	11.0
Q. J. R.	Negro	106	258	150	395	197	10.5
R.	Negro	107	237	I	850	795	17.4
R.	Negro	113	226	I	1,015	821	14.7
Q.	Negro	116	225	289	960	700	11.4
Q.	Negro	118	192	312	1,465	720	10.7
Max.		163	258	312	1,465	821	17.4
Min.		106	174	I	166	193	9.1
Ave.		134	219	--	698	500	12.0

*All values are based on 24-hour urine samples and volatile phenols as *p*-cresol and aromatic hydroxy acids as *p*-hydroxyphenylacetic acid.

†Insignificant trace.

Urinary volatile phenols, probably mainly *p*-cresol,[11] ranged from 91 to 302 mg., average 153 mg., and 106 to 163 mg., average 134 mg., calculated as *p*-cresol per 24 hours, for the urines from the white and Negro subjects.

While, therefore, individual urines show wide variations in the concentration of the various aromatic acids and phenolic bodies, no characteristic or outstanding racial difference between the two groups of urines appears to be present.

SUMMARY

Methods are described for the microbiologic determination of phenyllactic, *p*-hydroxyphenyllactic, and indole-3-lactic acids in urine. The volatile phenolic

and aromatic hydroxy acid fractions were determined chemically with the Folin-Ciocalteau reagent. While individual urines usually showed wide variations in these constituents, no obvious chemical difference between the urines from the white and Negro subjects appeared to be present.

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PLASMA 17-HYDROXYCORTICOSTEROIDS IN HYPERFUNCTION, SUPPRESSION, AND DEFICIENCY OF ADRENAL CORTICAL FUNCTION

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CONSIDERABLE advances have been made during the last decade in the understanding and evaluation of adrenocortical function in man. The determination of the 17-ketosteroid concentration in the urine has served as a useful tool in the clinical appraisal of endocrine abnormalities for nearly 20 years. The availability of adrenocorticotropin (ACTH) and the introduction of new methods for the estimation of steroids in body fluids, however, has made it possible to evaluate adrenocortical function more reliably. An important advance was the introduction by Thorn and associates[1] of the response of blood eosinophils to the administration of ACTH as a test of adrenocortical function. Subsequently, Thorn and co-workers[2] reported that the changes in the urinary excretion of 17-ketosteroids and 17-hydroxycorticosteroids were more direct indices of adrenocortical function. Recently, the development of methods for the determination of free blood 17-hydroxycorticosteroid (17-OHCS) levels has made it possible to study more precisely changes in adrenocortical activity and in the metabolism of 17-OHCS.[3, 4] The measurement of free plasma 17-OHCS reveals the concentration of corticosteroids before they are extensively metabolized, conjugated, and excreted in the urine.[3, 4]

In this paper we wish to present data on plasma 17-OHCS levels and their changes during ACTH or steroid administration in: (a) patients who had been on prolonged steroid therapy; (b) patients with adrenal insufficiency; and (c) patients with Cushing's syndrome. In addition, the metabolism of C^{14}-labeled cortisol (hydrocortisone) was studied in some of the subjects.

METHODS AND MATERIALS

Plasma 17-OHCS levels were determined in the early part of the study by the method of Nelson and Samuels[3, 4] and, more recently, by the method of Silber and Porter[5] as modified by Eik-Nes.[6]

Plasma 17-OHCS levels on patients who had been on prolonged cortisone (or cortisol) therapy were determined on bloods drawn following cessation of such therapy. The last dose of steroid was taken not later than 6 P.M. and the blood samples were obtained between 9 and 10 A.M. of the following day. The ACTH tests and the method of

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Received for publication Feb. 11, 1957.
*Work performed during tenure of a Scholarship in Cancer Research (1953-1956) of the American Cancer Society.
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administration of various steroids have been described previously.[7, 8] The methods for the administration of 4-C^{14}-cortisol and determination of radioactivity have been described in a previous publication.[9] Patients with known Addison's disease were taken off their cortisone or cortisol therapy for a minimum of 5 days before the test but were maintained on DOC therapy.

RESULTS

1. *Patients Studied After Abrupt Cessation of Steroid Administration.*—The levels of plasma 17-OHCS over a 6-hour period (9 A.M. to 3 P.M.) in a group of 12 patients who had been on prolonged cortisol or cortisone therapy, followed by abrupt cessation of the therapy the day before the blood samples were obtained, are compared in Fig. 1 with the values obtained in a group of 16 normal subjects. The mean values for the patients who had been on

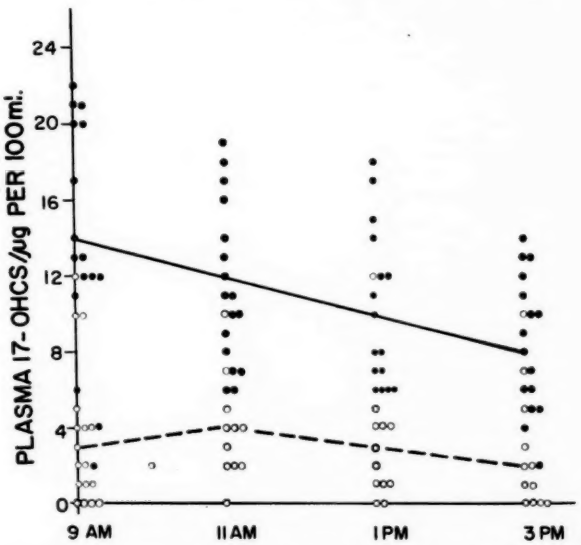

Fig. 1.—Plasma 17-OHCS levels in patients (open circles) following abrupt cessation of long-term steroid therapy the day before the sampling and in normal subjects (closed circles) from 9 A.M. to 3 P.M. The solid and broken lines represent the means of the plasma 17-OHCS in the normal subjects and in the steroid treated patients, respectively.

steroid therapy are much lower than those of the control group. Most of the values (0 to 4 μg/100 ml. of plasma) in the treated subjects were below the level of dependability of the method employed and are difficult to determine accurately by the method used.[3, 4] Some of the patients had values well within the normal range, even though these subjects had been on high doses of steroids for prolonged periods of time. The gradual decline of 17-OHCS levels in the control group reflects the normal diurnal variation of these 17-OHCS.[10]

In order to evaluate adrenocortical capacity in patients who had been on prolonged steroid therapy, a standard ACTH test was performed by infusing 40 I.U. of ACTH intravenously over 6 hours.[7] In Fig. 2 are shown the changes in plasma 17-OHCS levels during the infusion of ACTH. It is apparent that 5 of the patients had a subnormal response. The 17-OHCS rose normally in one patient, a 66-year-old woman with a disease suggestive of lupus erythematosus who had been on oral cortisone acetate therapy (50 to 150 mg. every

day) for at least a year. In Fig. 3 are shown the changes in plasma 17-OHCS levels in a male patient (L. C.) with rheumatoid arthritis given ACTH on 4 successive days following cessation of long-term cortisone acetate therapy.

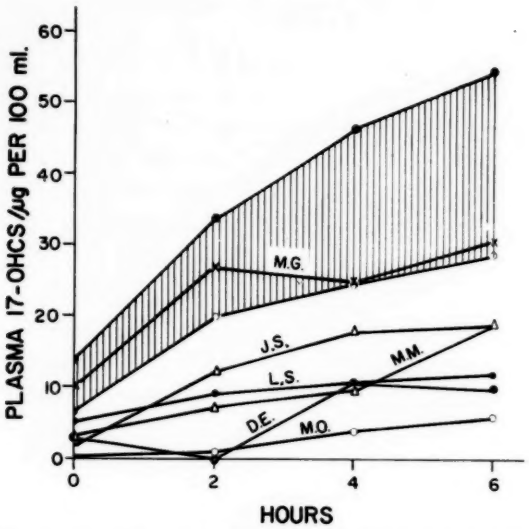

Fig. 2.—Changes in plasma 17-OHCS levels during the infusion of 40 units of ACTH in 6 subjects following abrupt cessation of prolonged steroid therapy. Shaded area represents range of response in normal subjects.

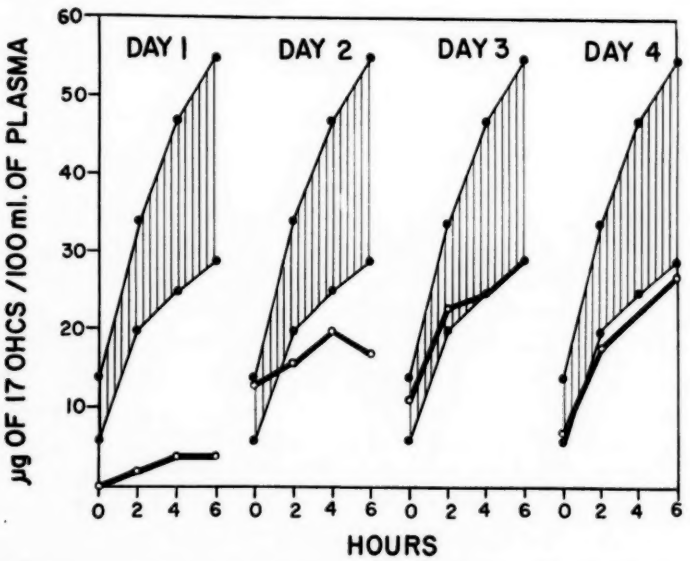

Fig. 3.—Changes in plasma 17-OHCS of Subject L. C. (40-year-old male), a patient with rheumatoid arthritis treated with cortisone acetate (250 mg. daily orally) for 6 months. On four successive days, following sudden discontinuation of the steroid therapy, ACTH (40 units) was given intravenously over 6 hours and the changes in plasma 17-OHCS were determined. The shaded areas represent the range of normal response.

On the first day the response was definitely subnormal, similar to the 5 patients in Fig. 2, on the second day it was slightly more adequate although still not normal, and on the third and fourth days the responses approached

normal. It should be noted that the changes in the plasma 17-OHCS response in Patient L. C. were probably influenced by the daily administration of ACTH.

One patient (W. N.), a 32-year-old woman with acute leukemia, had been on 200 mg. of cortisone acetate for one month, followed by gradual discontinuance of the steroid therapy over a period of several days, with the last dose of 25 mg. cortisone acetate being taken on March 31. On April 1 the patient's plasma 17-OHCS levels were 7 μg at 9 A.M., 9 μg at 10 A.M., and 4 μg at noon. The evening of that day the patient developed severe chills, fainted, and was hospitalized for septicemia. On April 2 her plasma 17-OHCS were 42 μg at 9 A.M., 39 μg at 10 A.M., and 27 μg at noon. In this patient rapid recovery of adrenocortical capacity under stress occurred even after steroid therapy of several weeks' duration.

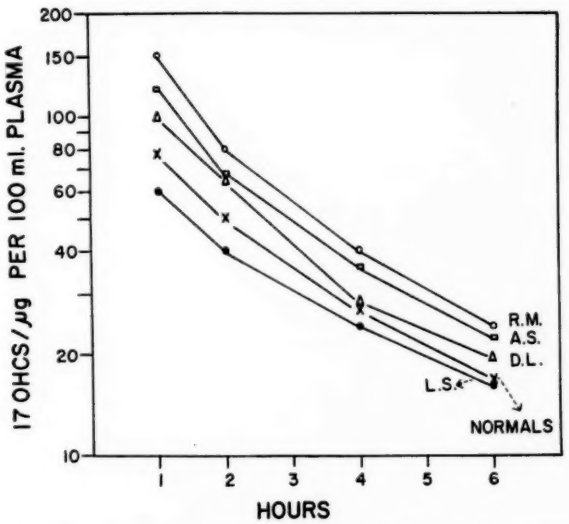

Fig. 4.—Clearance of 17-OHCS from plasma following the intravenous injection of 50 mg. of cortisol into subjects following abrupt cessation of long-term cortisone or cortisol therapy. The results are compared to the average of normal subjects. The control plasmas of the patients did not contain any demonstrable 17-OHCS levels.

One possible explanation for the low plasma 17-OHCS levels in patients after long-term administration of steroid compounds is that the prolonged administration of steroids might alter the metabolism of 17-OHCS. In order to test this possibility, 4 patients who had been on long-term, high-dose therapy of cortisone acetate received 50 mg. of cortisol intravenously on the day following abrupt cessation of treatment, and the clearance of 17-OHCS from their plasma was determined. The results are shown in Fig. 4. In none of the 4 subjects were concentrations of 17-OHCS determinable in the control plasma samples. The clearance rates in the patients were similar to those of normal subjects, even though in 2 patients (R. M. and A. S.) the concentrations were significantly higher than those seen in normal subjects. The amount of cortisol administered to the patients and the normal subjects was approximately twice the physiologic daily requirement, however, and although the

results do not support the hypothesis of altered metabolism, they do not disprove it. To pursue this proposition further, tracer amounts of C^{14}-labeled cortisol (1 μc/245 μg) were given to 2 patients following sudden cessation of

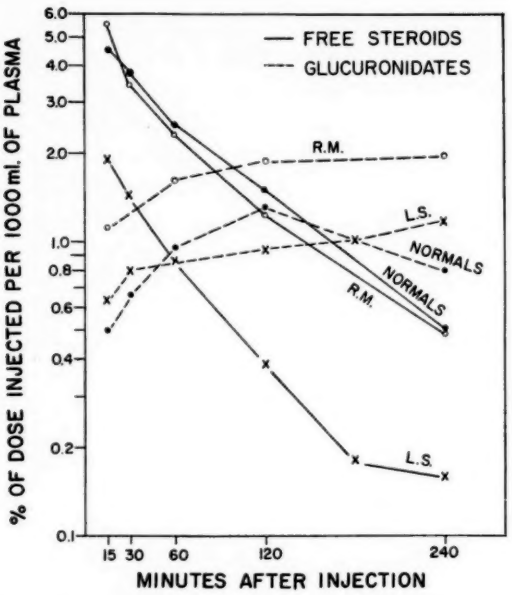

Fig. 5.—Radioactivity in the plasma free-steroid fraction and in the glucuronidate fraction of normal subjects and two patients, who had been on prolonged steroid therapy, following the injection of C^{14}-cortisol. Patient L. S. had the nephrotic syndrome with generalized, severe edema complicating generalized lupus erythematosus at the time of the study.

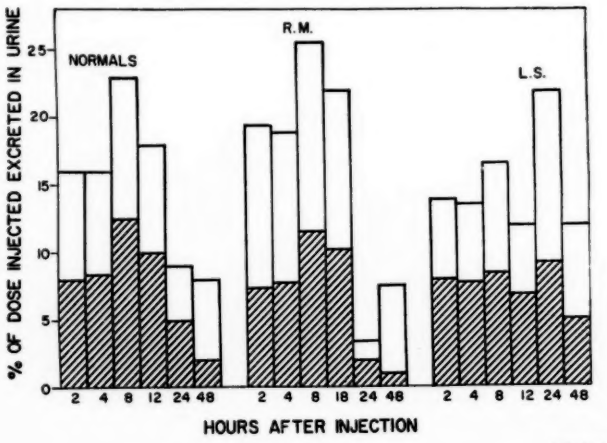

Fig. 6.—Urinary radioactivity following the injection of C^{14}-cortisol to same subjects as in Fig. 5. Shaded areas represent the steroids released by β-glucuronidase hydrolysis.

prolonged steroid therapy and the results compared to the mean of 5 normal subjects. The plasma levels of radioactivity readily extractable by chloroform (free steroids) and those of radioactivity released by β-glucuronidase hydrolysis (glucuronidates) are shown in Fig. 5. The rate of clearance of

free steroids was essentially normal in Subject R. M. The much lower concentrations of "free" activity in Patient L. S. may have been due to severe, generalized edema present at the time in this patient. In Fig. 6 are shown urinary concentrations of radioactivity in these patients. None of the subjects excreted more than 3 per cent of the radioactivity administered in the free form.

2. *Adrenocortical Insufficiency.*—Standard ACTH tests of adrenocortical capacity were performed in a group of patients with known or suspected Addison's disease. In Table I are shown the 17-OHCS levels in the plasma

TABLE I. PLASMA 17-OHCS DURING 6-HOUR ACTH INFUSION IN SUBJECTS WITH ADRENAL INSUFFICIENCY

SUBJECT	AGE AND SEX	μG 17-OHCS/100 ML. OF PLASMA DURING ACTH INFUSION				MAINTENANCE MEDICATIONS		DURATION OF DISEASE (YEARS)
		0 HR.	2 HR.	4 HR.	6 HR.	CORTISONE ACETATE MG./24 HR. TAKEN ORALLY	DOC TMA*	
M.	51 M	0.4	0.4	0.4	0.3	37.5	20	1
Ste.	35 M	0	0	0	0	25	30	5
H.	50 F	0.8	0.9	1.3	2.0	25-37.5	25	13
Sto.	45 F	0	0	0	0	37.5	30	3-4
Cl.	30 F	0.9	1.9	2.1	1.0	25	20	12
W.	47 M	0	0	0	0	12.5	40	12
Sw.	40 F	0.2	2.9	3.7	3.2	37.5	40	5
T.	33 M	0.3	1.0	0.9	0.7	25	40	16
B.	19 F	0	0	0	0	37.5	50	6
N	53 F	0	0	0	0	25	62	16
Ch.	19 M	1.7	1.6	2.9	5.2	25	25	1
R.	64 M	4.0	1.3	1.8	1.7	37.5	30	1
Normals		11	27	35	40			

*Deoxycorticosterone trimethylacetate.
For explanation of underlining see text.

of patients with proved Addison's disease during the administration of ACTH. The results are correlated with some of the clinical data. For complete interpretation of the results in this table it should be stressed that the plasma 17-OHCS were determined by the method of Silber and Porter[5] as modified by Eik-Nes.[6] The underlined levels of 17-OHCS in Table I are from samples exhibiting a peak absorbancy at 410 mμ in the Porter-Silber reaction,[11] similar to the absorbance of 0.5 μg of crystalline cortisol taken through the entire procedure. This peak was not present in the remaining (not underlined) samples. The peaking at 410 mμ is highly indicative that the measured levels are due to the presence of minute quantities of steroids exhibiting a 17, 21-dihydroxy-20-ketone side chain (cortisol and some of its metabolites). The

method used[6] for the determination of the 17-OHCS levels will not measure accurately concentrations of 17-OHCS below 1.2 μg/100 ml. of plasma. The range for normal subjects between 9 A.M. and 2 P.M. is 7 to 15 μg/100 ml. of plasma, the average being 10 μg.

From Table I it can be seen that none of the patients with Addison's disease had control values of plasma 17-OHCS within the normal range, and only 2 of the control values fell within the reliable range of the accuracy of the method. In 4 of the subjects the administration of ACTH resulted in the appearance in plasma of cortisol-like substances (underlined values) as indicated by their absorption at 410 mμ. None of the patients, however, showed a response to ACTH which approached that of normal subjects. There was no apparent correlation between the clinical picture of these patients and the appearance of cortisol in the plasma.

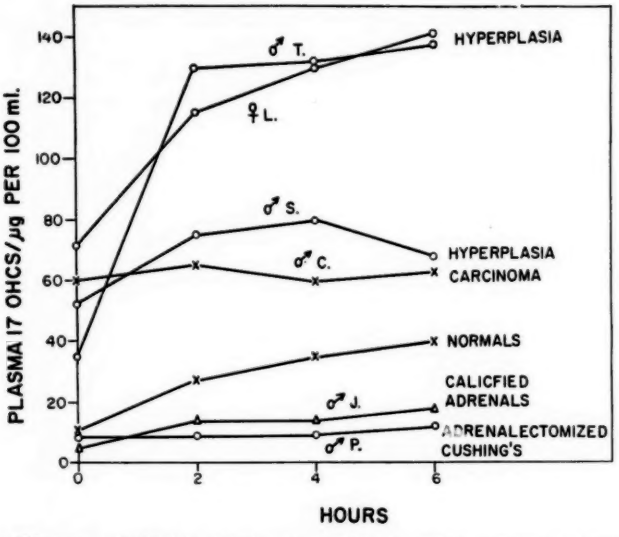

Fig. 7.—Plasma 17-OHCS levels in subjects with adrenal disease. Note lack of response of patient with carcinoma of the adrenal gland and exaggerated responses of patients with adrenal hyperplasia.

In Fig. 7 are shown the changes in the plasma 17-OHCS levels in a subject (J.) with calcification of both adrenal glands. This 30-year-old man had noticed definitely increased pigmentation of his skin and salt-craving. The response of the 17-OHCS to the administration of ACTH is definitely subnormal and may indicate partial adrenal insufficiency.

3. *Adrenal Hyperfunction.*—Changes in plasma 17-OHCS levels during the intravenous infusion of ACTH were determined in 4 patients with Cushing's syndrome. The results are shown in Fig. 7. In all the cases the control plasma 17-OHCS levels were repeatedly much higher (35 to 82 μg) than those seen in normal subjects (7 to 15 μg).

Two of the patients with adrenal hyperplasia (T. and L.) showed responses to the infusion of ACTH which were of much greater magnitude than those seen in normal subjects. The response of Patient S. with adrenal hyperplasia was not as prominent as that seen in Patients T. and L., and was similar

to the response of normal subjects. No significant changes in the plasma 17-OHCS levels occurred in the male subject with Cushing's syndrome due to carcinoma of the left adrenal gland with metastases to the lung. Subject P. was a man with Cushing's syndrome due to bilateral adrenal hyperplasia for which bilateral total adrenalectomy had been performed. More than a year after the operation the plasma from this patient contained normal levels of 17-OHCS, which did not rise significantly when ACTH was administered. On another occasion, when the patient was taking 0.5 mg. of fluorohydrocortisone 4 times daily, and his urinary 17-OHCS ranged from 0.70 to 1.05 mg. per day, the administration of 80 units of ACTH daily for 5 days resulted in a rise of the urinary 17-OHCS, with the first day's excretion being 1.84 mg. and the fifth day's 4.23 mg. These findings indicate that even though bilateral adrenalectomy had been performed on this patient, some functioning adrenocortical tissue was still present in the patient, although it was sluggish in its response to ACTH.

DISCUSSION

That the administration of adrenal steroids will cause atrophy and hypofunction of the adrenal cortex of animals has been known for many years.[12] These findings have been corroborated in human subjects during the administration of therapeutic doses of cortisol or cortisone.[13, 14] The results of the present study, based on plasma 17-OHCS levels, confirm the profound depression of adrenocortical capacity produced by long-term steroid therapy but also indicate the considerable individual variation in the speed with which adrenocortical function may return to normal. Examination of the experimental and clinical data fails to reveal any significant correlation between the dose of steroid taken, the length of time of steroid therapy, the depression of the plasma 17-OHCS levels, and the response of the plasma 17-OHCS to ACTH. In one subject (L. C.) the response of the plasma 17-OHCS did not return completely to normal even after 4 successive daily intravenous administrations of ACTH, whereas Subject M. G. showed a fairly normal response during the first day. Following gradual reduction and then discontinuation of cortisone therapy, Patient W. N. also had a normal elevation of 17-OHCS levels following the stress of an acute infection.

The rates of metabolism of a 50 mg. dose of cortisol in patients who had been on prolonged and intensive steroid therapy did not differ significantly from that observed in control subjects. The metabolism of 4-C^{14}-cortisol was essentially normal in one of the two patients who received a tracer dose of the radioactive steroid. The other subject (L. S.), at the time the radioactive cortisol was injected, had severe, generalized edema and a clinical picture compatible with the nephrotic syndrome complicating lupus erythematosus. The lower levels of radioactivity in the plasma free steroid-fraction may be related to migration of C^{14}-cortisol into the large extravascular space. Previous studies have shown that 17-OHCS may appear in extravascular fluids.[15] Altered metabolism, however, of C^{14}-cortisol cannot be ruled out in Patient L. S.

From the data presented it is apparent that most patients with definite clinical evidence of Addison's disease do not have significant amounts of 17-OHCS in the plasma, even after maximal stimulation with ACTH. In several patients, however, the administration of ACTH resulted in low but definite concentrations of 17-OHCS in plasma indicating the presence of small amounts of adrenocortical tissue. Most, but not all, patients with Addison's disease have been shown to have demonstrable amounts of ACTH in their blood, whereas this is not so for most normal subjects and for patients with Cushing's syndrome.[16-18] The presence of demonstrable ACTH activity in the bloods of most subjects with adrenal insufficiency has been postulated to be due to lack of circulating corticosteroids which regulate and inhibit ACTH production by the pituitary. In patients with Addison's disease who may have small amounts of functioning adrenocortical tissue one might expect maximum stimulation of the tissue by the high circulating levels of endogenous ACTH and hence no response of the plasma 17-OHCS levels to the exogenous ACTH. Nevertheless, in 4 of the patients of the present study definite, albeit subnormal, elevations in circulating 17-OHCS were seen during the ACTH infusions. It is possible that these subjects did not have elevated endogenous blood ACTH levels, and hence, the small remnants of adrenocortical tissue responded to the large amounts of exogenous ACTH. In this connection, it is interesting to note that Addison's disease due to unitropic (ACTH) deficiency of the pituitary gland has been reported recently.[19] It is possible that some of the subjects in the present study who responded partially to ACTH may suffer from such a deficiency.

The existence of partial adrenal insufficiency is indicated by the subnormal response to ACTH of some of the patients who had been on prolonged corticosteroid therapy and by Subject J. A similar picture of partial adrenocortical insufficiency has been described previously.[7, 20]

The changes in the plasma 17-OHCS of patients with Cushing's syndrome produced by the administration of ACTH indicate that in most cases of adrenocortical hyperplasia the response is exaggerated, occasionally to an extreme degree. On the other hand, in the patient with carcinoma of the adrenal no significant changes in the plasma 17-OHCS were apparent. A previously published report indicated that of 3 cases with Cushing's syndrome the subject with adrenocortical carcinoma exhibited an "inflexible, autonomous type of increased steroid production and excretion unaffected by ACTH."[21] On the other hand, the patient with an adrenocortical adenoma and the subject with adrenal hyperplasia exhibited a "flexible type of increased corticosteroid production and excretion which responded in an exaggerated manner to ACTH stimulation."[21] Recently, one of us[22] made a review of the cases of Cushing's syndrome followed in several clinics. The effect of ACTH administration (25 I.U. intravenously over 4 to 6 hours) on plasma and/or urinary 17-OHCS was studied in 56 cases of bilateral adrenal hyperplasia, 11 cases of unilateral benign adenoma, and 11 cases of adrenocortical carcinoma. In the hyperplasia group, 51 patients exhibited a hyperresponse, 4 a normal response, and 1 no significant change. Of the 11 cases of adenoma, 8 showed

a hyperresponse, 1 a normal response, and 2 no change. Finally, 2 patients with carcinoma of the adrenal cortex had a hyperresponse, 1 a normal response, and 8 did not respond.

From the results presented it would appear that the determination of the plasma 17-OHCS levels may give us important information in the evaluation of adrenocortical function. The capacity of the adrenal cortex is not an all-or-none phenomenon, but one showing considerable variation from subject to subject and within one clinical entity, e.g., adrenal insufficiency or hyperfunction.

SUMMARY

Plasma 17-OHCS levels and adrenocortical capacity were greatly reduced in most patients on long-term steroid (cortisol, cortisone) therapy. In some subjects, however, the adrenal cortex showed remarkable ability in returning to normal function after abrupt cessation of steroid therapy. The rate of metabolism of intravenously administered cortisol was essentially normal in subjects following abrupt cessation of prolonged steroid therapy, although the plasma 17-OHCS concentrations tended to be higher in 2 of the patients. Four of 12 patients with Addison's disease had low levels of 17-OHCS during ACTH infusion. Two of 3 patients with Cushing's syndrome due to adrenocortical hyperplasia showed exaggerated plasma 17-OHCS levels during ACTH infusion, while the third patient had a fairly normal response. A patient with functioning carcinoma of the adrenal showed elevated 17-OHCS levels with no changes during ACTH infusion.

We wish to thank the Endocrinology Study Section of the National Institute of Health for generously supplying the C^{14}-cortisol.

Thanks are due to Mr. Lawrence Beecher and Mrs. Kari Brinck-Johnson for technical assistance.

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LABORATORY METHODS

A COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF SERUM GLUTAMIC OXALACETIC TRANSAMINASE

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THE possible clinical significance of an increased serum glutamic oxalacetic transaminase (GOT) level, was reported by La Due, Wroblewski, and Karmen.[4, 8] Numerous publications have since appeared in which the transaminase activity has been employed as a diagnostic tool[7, 9, 13]; increased serum transaminase activity has been reported in pathologic conditions involving myocardial infarction,[1, 6, 8] hepatocellular damage,[1, 13] obstructive jaundice,[1] and acute pancreatitis.[1]

Karmen[5] devised a spectrophotometric method for determining GOT activity. The assay is based on the transamination of aspartic acid with α-ketoglutaric acid to form oxalacetic acid and glutamic acid (Equation I); as oxalacetic acid is formed, it is reduced to malic acid in the presence of exogenous malic dehydrogenase and reduced diphosphopyridine nucleotide ($DPNH^+$) (Equation II). The amount of $DPNH^+$ oxidized to DPN is determined with an ultraviolet spectrophotometer and expressed as transaminase units.

Equation I:

$$\underset{\text{Aspartic Acid}}{HOOC\cdot CH_2\cdot CHNH_2\cdot COOH} + \underset{\alpha\text{-Ketoglutaric Acid}}{HOOC\cdot CH_2\cdot CH_2\cdot CO\cdot COOH} \underset{\text{Phosphate Buffer pH 7.4}}{\overset{\text{Transaminase}}{\underset{37^\circ\text{ C.}}{\rightleftharpoons}}} \underset{\text{Oxalacetic Acid}}{HOOC\cdot CH_2CO\cdot COOH} + \underset{\text{Glutamic Acid}}{HOOC\cdot CH_2\cdot CH_2\cdot CHNH_2\cdot COOH}$$

Equation II:

$$\underset{\text{Oxalacetic Acid}}{HOOC\cdot CH_2CO\cdot COOH} + DPNH + \underset{\text{Co I } H_2}{\overset{\text{Malic Dehydrogenase}}{\underset{\text{Phosphate Buffer}}{\rightleftharpoons}}} \underset{\text{Malic Acid}}{HOOC\cdot CH_2CHOH\cdot COOH} + \underset{\text{Co 1}}{DPN}$$

Equation III:

$$\underset{\text{Oxalacetic Acid}}{HOOC\cdot CH_2\cdot CO\cdot COOH} \xrightarrow[37^\circ\text{ C.}]{\text{Aniline-Citrate}} \underset{\text{Pyruvic Acid}}{CH_3\cdot CO\cdot COOH} + CO_2$$

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Received for publication Nov. 26, 1956.

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The method of assay described in this paper does not involve the use of an ultraviolet spectrophotometer or DPN. It is a modification of the procedure described by Tonhazy, White, and Umbreit[12] for the determination of transaminase activity in tissue.* The assay is similar to the Karmen method in that the same basic transamination reaction (Equation I) is involved. However, the reduction of oxalacetic acid to malic acid (Equation II) is replaced by the conversion of oxalacetic acid to pyruvic acid by aniline-citrate (Equation III), and a subsequent quantitative determination of pyruvic acid by the formation of its 2,4-dinitrophenylhydrazine derivative in a manner similar to that described by Friedemann and Haugen.[2]

REAGENTS AND EQUIPMENT

1. Buffer, pH 7.4 (0.1 M): Sodium Phosphate Buffer (2.30 Gm. $Na_2HPO_4 \cdot H_2O$ and 11.83 Gm. NaH_2PO_4 [anhydrous] dissolved in water and diluted to one liter).

2. Sodium Aspartate (0.2 M): 0.665 Gm. DL-aspartic acid dissolved in 20 ml. of above buffer, pH adjusted to 7.4 with 2.5 N KOH and diluted with buffer to 25 ml. Small portions of this and the following solution were placed in tubes and stored at –20° C.

3. Sodium α-ketoglutarate (0.1 M): 0.365 Gm. α-ketoglutaric acid dissolved in 20 ml. of buffer pH adjusted to 7.4, with 2.5 N KOH and with buffer diluted to 25 ml.

4. Ten per cent metaphosphoric acid: 10 Gm. metaphosphoric acid dissolved in water and diluted to 100 ml.

5. Aniline-citrate: 100 Gm. citric acid dissolved in water and diluted to 100 ml. Immediately prior to use equal amounts of C.P. aniline, which was not redistilled, and citric acid were mixed together.

6. 2,4-dinitrophenylhydrazine reagent (0.1 per cent): 0.1 Gm. 2,4-dinitrophenylhydrazine dissolved in 20 ml. concentrated (37 per cent), HCl, diluted to 100 ml. with water.

7. Water saturated toluene: 100 ml. of reagent grade toluene shaken thoroughly with 10 to 15 ml. of water in a separatory funnel. The excess water is removed, and the toluene is stored in a brown bottle, at 4° C.

8. Alcoholic KOH (2.5 per cent): 2.5 Gm. KOH dissolved and diluted to 100 ml. with 95 per cent ethanol. This was prepared daily.

9. Sodium Pyruvate Standard: Sodium pyruvate was prepared according to Robertson.[10] Ten milliliters of redistilled pyruvic acid, dissolved in 100 ml. ethanol, was neutralized with alcoholic NaOH (10 ml. water saturated NaOH diluted with 100 ml. ethanol) with constant stirring in an ice bath. Sodium pyruvate precipitated out as a white amorphous powder. This precipitate was collected on a Buchner funnel and washed four times with 50 ml. of cold ethanol, and twice with 25 ml. of cold ethyl ether. The white powder was dried over calcium chloride for 18 hours in a vacuum desiccator. A stock solution containing 800 mcg. of pyruvic acid per milliliter was prepared by dissolving 100 mg. of sodium pyruvate in 100 ml. 0.1 M PO_4 buffer pH 7.4.

10. Photoelectric Colorimeter with 450 mμ filter.

PROCEDURE

The enzyme assay was performed in the following manner: To 2 (18 × 150 mm.) test tubes, the following were added:

TEST	*BLANK* (Zero Time Control)
0.2 ml. serum	0.2 ml. serum
0.3 ml. buffer	0.3 ml. buffer
	0.3 ml. 10 per cent metaphosphoric acid
0.5 ml. DL-aspartate	0.5 ml. DL-aspartate

*Following the development of this method, we were informed that Dr. W. W. Umbreit adapted the Tonhazy, White, and Umbreit procedure for the determination of serum glutamic oxalacetic transaminase.

Mix and incubate for 30 minutes
at 37° C.

0.2 ml. α-ketoglutarate 0.2 ml. α-ketoglutarate

Mix and incubate for 10 minutes
at 37° C.

0.3 ml. 10 per cent metaphosphoric acid ----------

Two drops of aniline citrate are added to each tube, and incubated for 10 minutes at 37° C. One milliliter of the 0.1 per cent 2,4-dinitrophenylhydrazine reagent is then added to each tube, and the tubes again incubated for 5 minutes at 37° C. The tubes are removed from the water bath and 3.0 ml. of water saturated toluene is added, to extract the pyruvophenylhydrazine. The tubes and their contents are mixed vigorously, and then centrifuged for 5 minutes. The toluene layer is removed with a capillary pipette, and placed in a separate tube. One milliliter of the toluene extract is placed in a colorimeter tube

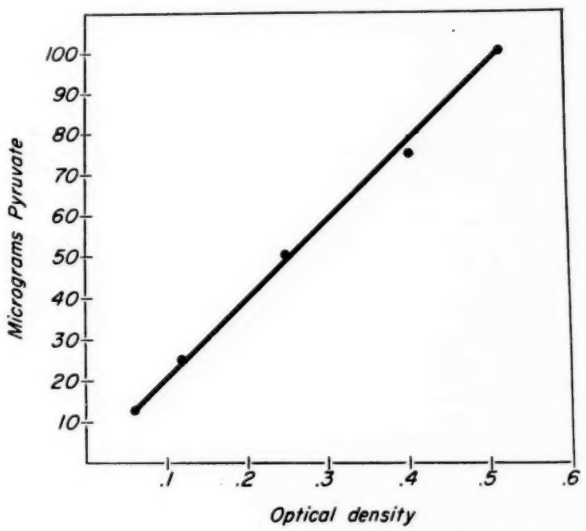

Fig. 1.—Relationship of sodium pyruvo-dinitrophenylhydrazine to optical density.

containing 5 ml. of 2.5 per cent alcoholic KOH solution and 0.5 ml. of water. The contents in the tube are mixed and allowed to stand at room temperature for 10 minutes. The tubes are read at 450 mμ in a Bausch and Lomb "Spectronic 20" photoelectric colorimeter. The colorimeter was zeroed (blanked out) with the zero time control, and the optical density obtained with the test was read against a standard pyruvic acid curve to determine the amount of pyruvic acid. A standard control, containing 100 mcg. of sodium pyruvate is incorporated in each series of determinations.

STANDARDIZATION

This method was standardized by the addition of varying amounts of sodium pyruvate, in 0.3 ml. quantities, to a zero time control in lieu of 0.3 ml. of buffer, and treated in a similar manner as the zero time control system. The standard was read against a zero time control, and a straight line relationship was found to exist between the concentration of sodium pyruvate and optical density. (See Fig. 1.)

CALCULATIONS

A Karmen unit is defined as the enzymatic activity of 1 ml. of serum which would oxidize $DPNH^+$ (Equation II) resulting in an optical density change of 0.001 at 340 mμ

in 1 minute. The Karmen procedure requires 200 meg. of $DPNH^+$ in a 3.0 ml. system, or 66.66 meg. per milliliter. The micromolar extinction coefficient of $DPNH^+$ being 6.22/ml.,[3] one micromol in 3 ml. would give an optical density value of 6.22 or 2.07/ml.

A serum containing, say, 500 Karmen units per milliliter would produce an optical density change of 5.000 in 10 minutes. This is equivalent to the formation of (5.000/2.07) 2.42 μM of pyruvate or (2.42 × 88) 213 meg. of pyruvate.

The outlined procedure requires 0.2 ml. of serum in a 1.5 ml. reaction system, which is equivalent to 0.4 ml. of serum in a 3.0 ml. reaction system. Four tenths milliliter of this serum, containing 500 Karmen units, would, therefore, produce (0.4 × 214) 85.2 meg. of pyruvate. Consequently 0.2 ml. of this serum in a 1.5 ml. reaction system would produce 42.1 meg. of pyruvate.

The following equation has been employed to convert the obtained optical density values to Karmen units:

$$\frac{\text{O. D. of Test}}{\text{O. D. of 42.1 meg. Pyruvate}} \times 500 = \text{Karmen units.}$$

RESULTS AND DISCUSSION

The validity and specificity of the Tonhazy, White, and Umbreit method for the determination of transaminase activity in serum was verified by employing complete, incomplete, and additive systems. Table I depicts the results obtained with such systems.

TABLE I. OPTICAL DENSITY VALUES OF PYRUVO-DINITROPHENOLHYDRAZONE OBTAINED WITH COMPLETE, INCOMPLETE, AND ADDITIVE SYSTEMS

SYSTEM*	OPTICAL DENSITY†	OPTICAL DENSITY‡
Inactivated serum§	.620	—
Serum + aspartate	.678	.056
Serum + α-ketoglutarate	.638	.022
Complete system	.796	.174
Complete system + 250 μg pyruvate	.905	.284
Complete system + 250 μg oxalacetate	.900	.278

*Reaction terminated with 0.3 ml. 100 per cent TCA.
†Colorimeter zeroed (blanked out) with water.
‡Colorimeter zeroed (blanked out) with inactivated serum.
§Serum inactivated with 0.3 ml. 100 per cent TCA.

Increasing the incubation time and/or the amount of serum yielded a proportional increase in the amount of pyruvo-dinitrophenylhydrazine. These data are presented in Table II. The addition of pyridoxal phosphate was without effect.

TABLE II. EFFECTS OF INCUBATION TIME AND VARIOUS AMOUNTS OF SERUM ON THE FORMATION OF PYRUVO-DINITROPHENOLHYDRAZONE

INCUBATION TIME (MIN.)	ML. OF SERUM	ML. OF BUFFER	PYRUVO-DINITRO-PHENOLHYDRAZONE (MCG.)
0.0	0.2	0.0	0.0
2.5	0.2	0.0	26.4
5.0	0.2	0.0	51.0
7.5	0.2	0.0	75.8
10.0	0.2	0.0	102.8
10.0	0.0	0.2	0.0
10.0	0.05	0.15	25.6
10.0	0.10	0.10	48.2
10.0	0.15	0.05	70.7
10.0	0.20	0.00	104.6

TABLE III. COMPARISON OF ZERO TIME CONTROL AND TESTS WITH DIFFERENT CONCENTRATIONS OF METAPHOSPHORIC ACID AND TRICHLOROACETIC ACID

SYSTEM	ACID—0.3 ML. (PER CENT)	OPTICAL DENSITY
Zero time control	100 Metaphosphoric	0.183
Test	100 Metaphosphoric	0.387
Zero time control	100 Trichloroacetic	1.398
Test	100 Trichloroacetic	1.699
Zero time control	10 Metaphosphoric	0.147
Test	10 Metaphosphoric	0.347
Zero time control	10 Trichloroacetic	0.830
Test	10 Trichloroacetic	1.032

TABLE IV. COMPARISON OF TRANSAMINASE ACTIVITY VALUES* OBTAINED WITH THE KARMEN METHOD, TONHAZY, WHITE, AND UMBREIT METHOD (TCA) AND OUR MODIFICATION (METAPHOSPHORIC ACID)

SERUM NO.	KARMEN†	TCA	METAPHOSPHORIC	DIAGNOSIS
1	0,2	11,15	0,0	Normal
2	3,5	11,20	5,8	Normal
3	7,9	15,20	10,13	Normal
4	25,28	40,48	27,33	Normal
5	38,40	56,63	40,41	Normal
6	40,42	60,75	41,45	Normal
7	53,55	70,82	54,58	Normal
8	57,59	71,80	60,65	Normal
9	60,63	75,94	60,68	Normal
10	70,74	93,105	75,80	Normal
11	55,57	80,93	59,62	Cirrhosis of liver
12	108,112	140,173	111,117	Cirrhosis of liver
13	35,38	61,78	39,43	Cirrhosis of liver
14	450,460	320,380	470,485	Acute hepatitis
15	790,805	530,610	819,840	Acute hepatitis
16	825	590	865	Chronic hepatitis
17	1090	635	1135	Chronic hepatitis
18	168	206	175	Hepatitis
19	605	425	638	Acute myocardial infarction
20	310	400	335	Acute myocardial infarction
21	184	250	196	Myocardial infarction
22	62	94	66	Myocardial infarction
23	73	100	77	Myocardial infarction
24	148	183	155	Myocardial infarction
25	130	174	136	Myocardial infarction
26	100	130	105	Myocardial infarction
27	191,197	242,278	203,209	Constrictive pericarditis (etiology, tuberculosis)
28	349	410	367	Rheumatic heart disease
29	236	276	241	Thorazine hepatitis
30	68	87	71	Pulmonary embolism with pleural effusion
31	15	31	19	Obstructing ulcer
32	93	150	100	Carcinomatosis
33	144,147	186,210	148,153	CA of ascending colon with hepatic metastasis
34	88,92	148,182	93,100	Grand mal epilepsy
35	150	193	155	Obstructive jaundice
36	18	48	22	Pneumonia
37	3	17	5	Pneumonia
38	60	88	64	Epilepsy
39	20	28	20	Multiple sclerosis
40	80	125	84	Bronchogenic carcinoma
41	160,165	215,258	168,175	Acute leukemia
42	27	52	31	Cerebral thrombosis

*All values are expressed as Karmen units.

†Preincubation period, as well as the 10-minute photometric analysis were performed at 37° C.

When the original method was checked against the Karmen procedure (performed at 37° C.) much larger and poorly reproducible values were obtained. This we believe was due to the intense chromogen formed with the 100 per cent trichloroacetic acid (TCA) (Table III). To compensate for this, 10 per cent metaphosphoric acid or 10 per cent TCA was used in lieu of the 100 per cent TCA and a 30-minute preincubation period with aspartic acid was instituted. Both of these modifications yielded results approximately 10 per cent higher than those obtained with the original Karmen procedure, as well as a reproducibility factor of approximately 5 per cent. Table IV depicts the transaminase activities of sera compared by the Karmen method, the Umbreit method, and our modification.

SUMMARY

A colorimetric procedure for the determination of serum glutamic oxalacetic transaminase, which does not require either an ultraviolet spectrophotometer or reduced DPN, is described. The procedure is a modification of that which has been employed by Tonhazy, White, and Umbreit. The following modifications were found necessary to insure a reproducible correlation between the amounts of sodium pyruvo-dinitrophenylhydrazine, and transaminase units as defined by Karmen:

1. The serum was preincubated with the aspartic acid for 30 minutes.

2. Ten per cent metaphosphoric acid or 10 per cent TCA, rather than 100 per cent trichloroacetic acid was employed to terminate the reaction.

We wish to thank Drs. Joseph L. Rabinowitz and H. M. Vars for their valuable advice, Dr. J. J. Boutwell for performing some of the determinations, and Dr. W. W. Umbreit for sending us a report of his procedure.

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VARIATION IN COMPLEMENT FIXATION ANTIGENICITY OF DIFFERENT YEAST PHASE STRAINS OF HISTOPLASMA CAPSULATUM

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INTRODUCTION

IN A recent study Schubert and his associates[1] demonstrated that strains of *Histoplasma capsulatum* differed in their ability to produce Histoplasmin of satisfactory antigenicity. The objective of the present investigation was to determine whether variations in potency occurred in a group of antigens derived from strains of *H. capsulatum* in the yeast phase.

MATERIALS AND RECORDS

Eleven strains of *H. capsulatum* were converted to the yeast phase by means of serial subcultures on cotton-plugged tubes of blood-free brain heart infusion agar* incubated at 37° C. Four-day-old yeast phase growths were harvested in sterile physiologic saline containing 0.01 per cent Merthiolate. After storage at room temperature for 48 hours, the suspensions were filtered through gauze, centrifuged, and the cells resuspended in Merthiolate-saline solution. The antigens then were adjusted volumetrically to produce a 10 per cent stock suspension of cells in barbital-buffered saline. Subsequently each yeast phase antigen was tested for anticomplementary activity as well as antigenicity by means of a box titration. The optimum dilution of antigen for each strain was selected on the basis of agreement noted in box titrations on several human sera from proved cases of histoplasmosis. Tests on rabbit sera were performed with optimum antigen dilutions as determined by box titrations with the homologous rabbit antisera.

The strains of *H. capsulatum* employed in this study and their sources are cited in Table I. All of the strains were recovered from human cases of histoplasmosis except for strain B11, which was isolated from a soil sample collected in a Peruvian cave by Lazarus and Ajello.[3]

Sera.—(1) Hyperimmune rabbit sera were prepared by injecting rabbits with suspensions of the yeast phase strains prepared in physiologic saline containing 0.3 per cent formalin and adjusted to a McFarland No. 3 density. Intravenous injections of 0.5, 1.0, 2.0, and 3.0 ml. of the yeast phase suspension were made at 4-day intervals. The rabbits were bled 7 days after the last injection.

(2) Human sera were obtained from culturally proved cases of histoplasmosis and from persons presumably infected by *H. capsulatum* as indicated by pulmonary lesions, clinical symptoms, and a positive Histoplasmin skin test. Sera were tested in serial dilutions beginning with a dilution of 1:8.

Complement Fixation Procedure.—The complement fixation procedure used was an adaptation of the 50 per cent end point technique, employing barbital-buffered physiologic saline throughout (Mayer et al.[2]). Antigens and serial dilutions of inactivated sera were

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Note: This is not an endorsement by the Public Health Service but rather a means of identifying a particular product.

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Received for publication Nov. 28, 1956.

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tested in 0.25 ml. of complement containing 5 fifty per cent units. Fixation was allowed to proceed for 15 to 18 hours at 4 to 6° C. The tubes then were placed at room temperature while the sensitized cells (a 2 per cent suspension of sheep erythrocytes sensitized for 10 minutes in the proportion of 1:1 with 4 units of hemolysin in 0.25 ml.) were prepared. The sensitized cells were added in 0.5 ml. amounts and the tests read after a secondary incubation period of 30 minutes in a 37° C. water bath. The titers were recorded as the highest dilutions of serum which gave a 3 plus or 4 plus reaction (30 per cent hemolysis or less).

TABLE I. SOURCE OF HISTOPLASMA CAPSULATUM CULTURES CONVERTED TO YEAST PHASE

STRAIN	SOURCE
CDC* Number	
28	N. F. Conant—1066; Duke University Medical School
105	N. F. Conant—1071; Duke University Medical School
267	Communicable Disease Center Collection
A228	M. L. Furcolow, Communicable Disease Center
A232	M. L. Furcolow, Communicable Disease Center
A233	M. L. Furcolow, Communicable Disease Center
A235	M. L. Furcolow, Communicable Disease Center
A811	C. W. Emmons—E6624; National Institutes of Health
A827	C. C. Campbell—G2; Army Medical School
B11	Communicable Disease Center Collection; Peruvian soil isolate
B12	S. B. Salvin—6515; National Institutes of Health

*Communicable Disease Center.

The complement binding power of the antigen was tested with 1.25, 2.5, and 5 fifty per cent units of complement. The antigen was considered to be anticomplementary when less than 80 per cent hemolysis was obtained with 2.5 fifty per cent units.

RESULTS

The comparative antigenicity of each yeast phase strain was tested first with the sera from immunized rabbits in order to determine whether the degree of reactivity obtained with rabbits could be used as a guide for selecting an antigen for the testing of human sera. The results obtained with human and rabbit sera are presented in Tables II and III. The results demonstrated that the 11 different strains of *H. capsulatum* developed antigens which showed wide ranges of activity, especially in tests with human sera (Table III).

TABLE II. COMPLEMENT FIXATION TITERS OBTAINED WITH ELEVEN STRAINS OF YEAST PHASE HISTOPLASMA CAPSULATUM AGAINST HOMOLOGOUS AND HETEROLOGOUS RABBIT ANTISERA

ANTIGENS		RABBIT SERUM ANTIBODY TITERS									
STRAIN	DILUTION*	28	105	A228	A232	A235	267	A811	B11	B12	A827
28	1:32	*2,048*	128	1,024	1,024	256	1,024	4,096	64	128	2,048
105	1:8	256	*32*	128	256	64	256	512	32	64	256
A228	1:32	1,024	64	*512*	1,024	256	512	4,096	32	128	1,024
A232	1:32	512	32	256	*512*	128	512	1,024	16	128	1,024
A233†	1:32	512	32	256	512	128	512	512	32	128	1,024
A235	1:32	1,024	64	512	1,024	*256*	512	1,024	32	256	2,048
267	1:32	512	16	256	256	128	*4,096*	1,024	16	64	2,048
A811	1:64	1,024	64	512	1,024	128	4,096	*1,024*	32	256	1,024
B11	1:64	64	0‡	128	256	64	256	512	*128*	32	128
B12	1:64	1,024	32	256	512	128	1,024	512	16	*128*	1,024
A827	1:16	512	64	256	512	256	512	1,024	32	256	*4,096*

Homologous antiserum shown by italics.
*Determined by box titrations with homologous sera.
†233 serum insufficient.
‡Less than 1:8.

In an attempt to grade the strains in respect to their antigenic activity, the data in Tables II and III were rearranged in the order of the median titers obtained with the human and rabbit sera tested. The results, presented in Table IV, show that the first 6 strains listed (28, A811, A228, A235, A827, and 232) gave higher median titers than the other 5 antigens and the orders of their listings were the same with both types of sera. Strain 28 gave the highest median titers and strain A811 gave the next highest titers with both groups of sera. The last two strains listed (105 and B11) were definitely inferior antigenically, giving the lowest median titers with both human and rabbit sera. The remaining three strains, 267, A233, and B12 were intermediary in position, giving definitely higher median titers than B11 and 105 when tested with human and rabbit sera.

TABLE III. COMPLEMENT FIXATION TITERS OBTAINED ON HUMAN SERA FROM CASES OF HISTOPLASMOSIS WITH 11 YEAST-PHASE HISTOPLASMA CAPSULATUM ANTIGENS

ANTIGENS		HUMAN SERUM ANTIBODY TITERS									
STRAIN	DILUTION*	1†	2	3	4	5	6‡	7	8	9	10
28	1:32	1,024	64	1,024	128	2,048	256	4,096	256	1,024	128
105	1:8	8	0§	0	8	32	16	16	0	32	8
A228	1:32	512	32	512	64	1,024	128	1,024	64	512	64
A232	1:32	512	32	512	64	512	64	512	128	512	64
A233	1:32	512	32	512	0	512	64	1,024	128	128	0
A235	1:32	512	16	512	64	1,024	128	1,024	128	512	64
267	1:32	512	16	512	128	512	128	2,048	128	512	128
A811	1:64	512	32	1,024	32	1,024	256	1,024	256	512	32
B11	1:64	0	0	0	0	8	16	16	0	0	0
B12	1:64	256	16	512	32	256	32	128	64	256	32
A827	1:16	512	32	512	64	512	64	512	128	512	64

*Determined by box titrations.
†Sera 1 to 5 from culturally proved cases of histoplasmosis.
‡Sera 6 to 10 from presumptive cases of histoplasmosis.
§Titers less than 1:8.

TABLE IV. ANTIGENICITY OF ELEVEN STRAINS ARRANGED IN ORDER OF THE MEDIAN TITERS OBTAINED WITH TEN HUMAN AND TEN RABBIT SERA

HUMAN SERA		RABBIT SERA	
STRAIN	MEDIAN TITER	STRAIN	MEDIAN TITER
28	512	28	1,024
A811	256-512	A811	512-1,024
A228	256	A228	512
A235	256	A235	512
A827	256	A827	256-512
A232	256	A232	256-512
267	256	A233	256-512
A233	128	B12	256-512
B12	64-128	267	256
105	8	105	128-256
B11	0	B11	128

DISCUSSION

As in the study with Histoplasmins, Schubert et al.,[1] the results of this investigation indicate that yeast phase suspensions of various strains of *H. capsulatum* vary in their antigenic potencies, which illustrates the necessity of screening a large number of strains in order to select antigens of the highest reactivity for use in serologic tests.

A comparison of the results obtained with homologous and heterologous antisera (Table II) showed that the highest titers were not necessarily produced with homologous antigens.

It is interesting to note that the results obtained in the box titrations of rabbit sera led to the use of the same optimum dilutions as had been determined by box titrations with human sera. Such an observation is contrary to the experience obtained with Histoplasmin antigens in which the optimum antigen dilutions generally are higher with hyperimmunized rabbit sera.

The data point out that the results obtained in various laboratories will be influenced by the particular strain from which the antigen is prepared. In a previous study designed to compare the antigenicities of a yeast phase and a Histoplasmin antigen prepared from a single strain, Schubert and his associates[4] selected strain 105 because it had proved satisfactory as a producer of a good Hisoplasmin antigen for use in complement fixation tests and because it could be converted readily into the yeast phase. The data presented here show that strain 105 produces a relatively poor yeast phase antigen, which may account for the low yeast phase titers reported in the previous study.

SUMMARY

The antigenicities of yeast phase suspensions derived from strains of *Histoplasma capsulatum* were determined through titrations of hyperimmunized rabbit sera and of human sera from culturally proved and presumptive cases of histoplasmosis. Considerable variations in reactivities were noted and the results obtained were similar with human or rabbit sera. Hyperimmune rabbit sera therefore may be used as control sera in the determination of the optimum yeast phase antigen dilutions. To assure the selection of potent yeast phase antigens for use in complement fixation tests a large series of antigens derived from different strains of *H. capsulatum* should be screened.

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SEPARATION OF BONE SODIUM, POTASSIUM, AND CALCIUM BY THE USE OF CATION EXCHANGE RESIN

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INTRODUCTION

During the last ten years an increasing interest in the determination of the concentration of sodium and potassium in bone has arisen, due to the recognition of the possibly important role of the bone system in the sodium and potassium metabolism of the body.[3, 10, 12] Evidence in support of this has been presented by many investigators.[2, 3, 5, 10, 11, 13]

The main difficulty in such determinations in cortical bone has been the extremely high concentration of calcium in relation to the concentrations of sodium and potassium. The relationship of calcium:sodium:potassium is approximately 200:4:1 when calcium is expressed as milligrams of the secondary phosphate and the other two as their chlorides.

The methods published up to 1955 for the analysis of sodium and potassium in bone can conveniently be divided into the following three groups. (1) Wet ashing in concentrated nitric acid, or dry ashing followed by solution of the ash in hydrochloric acid and repeated precipitations of the calcium in the bone sample as carbonate or oxalate. The sodium and potassium content is determined in the supernatant by either flame photometry or chemical methods (e.g., gravimetric zinc uranyl acetate). (2) Primary determination of the calcium content of the bone sample followed by flame photometry of a second aliquot with a comparable amount of calcium introduced as a blank in the standards. (3) Solution of the bone with immediate flame photometry of the mixture, disregarding the calcium content. Some investigators have found these methods satisfactory.[3, 11, 14] Others have found them unsatisfactory,[4, 6, 9] the main problems have been interference by the calcium in the flame photometry of the sodium, and large coprecipitation of sodium when calcium is precipitated before sodium determination is done.

In our hands the above methods were unsuitable when applied to artificial mixtures with known content of secondary calcium phosphate, sodium chloride, and potassium chloride in the proportions found in cortical bone. With the precipitation technique the recovery was only 85 per cent and too variable, method two was found to be an elaborate procedure, and method three gave falsely high values. Separation of sodium from potassium in the bone sample was not possible by any of these techniques. We then proceeded to develop the method described in this paper. During this time the publications of Forbes and D'Ambrúsio[7] and Forbes and Lewis[8] appeared. They separated

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Received for publication Dec. 27, 1956.

cations in bone with quantitative recovery of sodium by column chromatography, using a cation exchange resin (Dowex 50 × 12, 50 to 100 mesh). The bone samples were dissolved in 3 N HCl after preliminary drying and ashing in a muffle furnace.

Our method has many features in common with the method of Forbes, and for certain purposes it has advantages. The column used is smaller, and smaller bone samples can be examined with the same degree of accuracy owing to smaller volumes of eluate. It gives quantitative recovery of potassium in a separate fraction as well as of sodium. Preliminary drying and ashing of the bone is not necessary and this saves time and loss of material which is of importance when short-lived isotopes of sodium and potassium are employed (Na^{24} can be used instead of Na^{22}).[8]

MATERIALS AND METHODS

The cation exchange resin, Dowex 50, –400 mesh size, 12 per cent cross linked,* is made up in a column in a Pyrex glass tube of 12 mm. internal diameter and 20 cm. length. The lower end of the glass tube is melted to a conical shape with a narrow opening. In this

Fig. 1.—Diagram of column and container as described in text.

cone some glass fragments form the base for a layer of Pyrex glass wool, acting as support for the resin. On top of the resin there is a layer of loosely packed glass wool which assures an even and gradual flow into the resin. The eluting fluid which is to pass through the column is held in a 500 ml. container with an opening in the bottom suitable for the attachment of thin polyethylene tubing. The free end of this tubing is attached to a thin Pyrex tube which is placed through an airtight rubber stopper on top of the column tube. All parts are supported by suitable clamps to a 4-foot stand (Fig. 1).

An air space between the rubber stopper and the fluid above the resin permits the rate of flow to be controlled with sufficient accuracy by counting the drops that pass per unit time. Regulation of the rate of flow is easily undertaken by moving the 500 ml. container up or down, thereby avoiding the use of complicated equipment for this purpose.[1] The reservoir is large enough to secure a constant rate of flow during the separation period. Cessation of flow is obtained by a clamp on the polyethylene tubing.

*From Dow Chemical of Canada, Ltd., Montreal, Quebec.

The resin is immersed in distilled water, poured into the glass tube and allowed to settle to the required height. The height of the column will vary with different batches of resin, making it necessary to determine the elution curve of each batch (variation in height of column is from 10 to 15 cm.). The two elution curves shown (Figs. 2 and 3) are from two different batches. The resin is freed from unwanted cations by washing with 300 ml. of 3 N hydrochloric acid at a speed of 2 ml. per minute. Excess hydrogen ions are then removed by 200 ml. of distilled water. The column is then ready for use. A piece of bone of approximately 0.2 grams wet weight is the most satisfactory amount for a column of the size described. It should be freed from spongy bone, periosteum, and marrow. After weighing it is dissolved in 1 ml. of concentrated nitric acid by heating in a Pyrex test tube. When clear fluid is obtained the volume is made up to 10 ml. with distilled water and poured into the container, and an additional 10 ml. of water is used for washing of the test tube and added to the container. The 20 ml. of solution should fill the polyethylene tubing from the rubber stopper and up, before flow is started. It is also advisable to maintain a constant volume of distilled water on top of the resin prior to beginning each separation thereby avoiding changes in elution curves associated with variation in amount of fluid above the column. Normal hydrochloric acid is used as the eluting agent, with a rate of flow of 0.8 ml. per minute. The whole separation takes from 3½ to 4 hours. The sodium and potassium content of the eluate is determined in a Baird flame photometer.

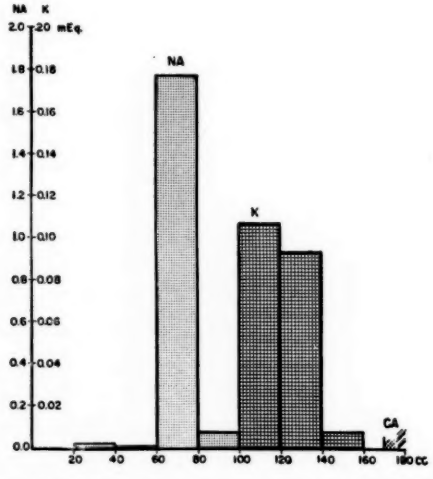

Fig. 2.—Elution curve for collection of sodium and potassium in same fraction (see text).

RESULTS

In Figs. 2 and 3 are shown two elution curves from bone biopsies. The first is useful when the removal of calcium is the only objective; the second shows the possibility of separating sodium and potassium in the same step as the removal of calcium. The volumes of eluate as it comes from the lower end of the column is plotted along the abscissa. The concentration of sodium and potassium in 10 c.c. samples are given in the ordinate. The potassium concentration is plotted in 10 times the scale of the sodium concentration, owing to the low concentration of the former. This gives a false impression of a significant overlap with potassium in the sodium fraction in Fig. 3. The actual content of potassium in the sodium fraction is only 0.00016 meq. The shaded area following the potassium peak is the spot where the first traces of calcium

occur as detected qualitatively with solutions of ammonium carbonate and oxalate. The interspaces between the fractions are sufficiently large to make the separation a workable procedure. The reproducibility of the pattern is, however, dependent on a high degree of standardization with the least possible variation in the different steps in the preparation and working of the columns. Rather than washing the calcium out of the resin with large amounts of acid for renewed use, we have chosen to use fresh resin for each separation.

During the actual use of the columns the eluate is not collected in 10 ml. samples, but the sodium and potassium fractions are taken out in single volumes. In the elution curve in Fig. 2, sodium and potassium is collected from 30 to 140 ml. In Fig. 3 the collection of sodium is from 30 to 90 ml., potassium from 100 to 160 ml.

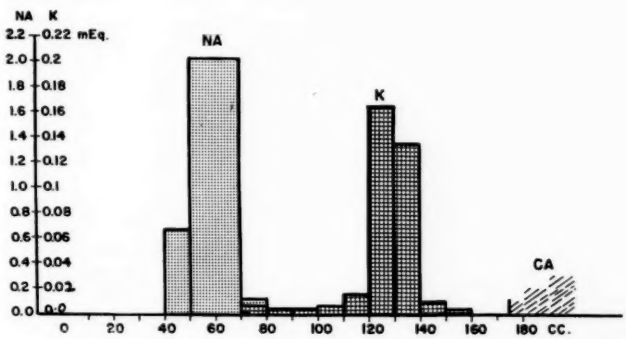

Fig. 3.—Elution curve for collection of sodium and potassium in separate fractions (see text).

The recovery rate in 6 consecutive experiments from artificial mixtures of secondary calcium phosphate, sodium chloride, and potassium chloride, with known amounts of cations simulating a 0.2 gram bone sample, was as follows:

(*a*) Sodium and potassium in one sample:

Content in sample	*Recovery*	*Deviation from true content*
Sodium 0.05 meq.	0.04913 ± 0.00055 meq.	Less than 3 per cent
Potassium 0.016 meq.	0.01511 ± 0.00017 meq.	Less than 6 per cent

(*b*) Sodium and potassium in separate samples:

Content in sample	*Recovery*	*Deviation from true content*
Sodium 0.05 meq.	0.050533 ± 0.00018 meq.	Less than 2 per cent
Potassium 0.01 meq.	0.00938 ± 0.000074 meq.	Less than 7 per cent

Attention should be paid to the extremely small standard deviations. The recovery of potassium is between 93 and 95 per cent, of sodium between 98 and 102 per cent.

DISCUSSION

In this laboratory more than 300 examinations of bone biopsies have been performed with the described technique. To date, none of the results indicates faulty separation or other technical difficulties. In most of the work radioactive sodium and potassium have been employed. Under these circumstances contamination with sodium in the potassium fraction would easily be detected.

One of the early difficulties with the resin was its impermeability to water when made up in a column. This was caused by a variation in size of the resin particles. A more homogeneous size of the particles is obtained if the resin is immersed in distilled water and allowed to settle by standing. The lighter and smaller particles will then parcipitate last and can be poured off. Repetition of this procedure a few times will secure an easily permeable resin column. A slight contamination with potassium may be encountered even in analytically pure hydrochloric acid. The amounts present are usually too small to give any significant error in the potassium determinations, but a periodic check of the acid is justified.

SUMMARY

A technically simple method is described for the separation of bone sodium, potassium, and calcium by the use of a cation exchange resin in a column. The recovery of sodium is between 98 and 102 per cent, of potassium between 93 and 95 per cent.

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THE CHLAMYDOSPORES OF CANDIDA ALBICANS: COMPARISON OF THREE MEDIA FOR THEIR INDUCTION

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Candida albicans, the most common etiologic agent of moniliasis, can be identified readily if cultures produce submerged mycelium bearing spherical clusters of blastospores and characteristic chlamydospores. Production of chlamydospores is favored by a reduced oxygen tension, limiting nutrients, and a temperature of 18° to 25° C. They have the following morphology: (1) generally spherical; exceptionally oval, lacrimoid, pyriform, or cylindrical; (2) enlarged in comparison with hyphae and blastospores, ranging in diameter from 6 to 17 μ, mostly 8 to 9 μ; (3) wall thick, hyaline, "double contoured"; (4) borne terminally, but may be lateral or intercalary; when intercalary, usually cylindrical; (5) sometimes sessile, but usually pedunculated and borne on a distinctive subterminal cell, the "chlamydospore"; (6) single or in short chains or clusters; (7) cytoplasm with one or more coarse, refringent droplets.

Since a limited supply of nutrients is essential for chlamydospore production, these structures will not generally be formed on the peptone-carbohydrate media, such as Sabouraud's glucose agar, employed for the isolation of *C. albicans* from clinical specimens.

For induction of chlamydospores, Benham recommended, in 1931, a corn meal infusion agar, a medium prepared by extracting 4 per cent corn meal in water at 60° C. for one hour, followed by filtration, reconstitution to original volume, and the addition of 1.5 per cent agar.

This corn meal infusion agar became widely adopted by many laboratories. The difficulties involved in preparing reproducible lots of this non-synthetic medium, however, soon became evident from the fact that chlamydospores were frequently not produced by strains otherwise fulfilling the criteria for *C. albicans*. Since determination of these other criteria requires tests of the fermentation pattern and sometimes also of animal pathogenicity, identification of *C. albicans* in the absence of chlamydospores becomes unnecessarily prolonged. Several investigators, therefore, have tried to standardize or simplify the preparation of corn meal infusion agar, or to devise other media to induce chlamydospore production in *C. albicans*.[1, 2, 5-9, 13-16]

Of the various new media proposed, two seemed to have certain advantages over corn meal infusion agar; one, the purified polysaccharide medium of Nickerson and Mankowski,[12] because of its "known composition" and the

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Received for publication Jan. 21, 1957.
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fact that it is available commercially*; the second, unpurified soluble-starch agar[4] because it is simpler to prepare and is more transparent than corn meal infusion agar, permitting easier examination of the submerged mycelium.

A comparison of these 3 media for chlamydospore-inducing ability was, therefore, conducted with freshly isolated yeasts and is being reported in this paper. The procedure for identifying 179 isolates included not only determinations of morphology on the above 3 media, but also a study of their fermentation pattern by methods to be described below.

MATERIALS AND METHODS

The 3 media compared in this experiment were prepared as follows:

1. Chlamydospore Agar (Difco). Rehydration of the powdered mixture with distilled water.

2. Soluble-starch agar. Addition of 1 per cent soluble starch* (Fehling-negative) to 2 per cent agar in distilled water.

3. Corn meal infusion agar. Prepared after Benham[3] from unenriched yellow corn meal obtained at a local bakery, and modified to increase the agar concentration to 2 per cent.

The 3 media were poured while still molten in 15 to 20 ml. amounts into standard, 10 cm. Petri dishes and allowed to solidify.

To provide conditions of slightly reduced oxygen tension, the plates were inoculated by cutting running streaks into the agar with the hammered bent tip of a stiff inoculating needle, taking care not to cut all the way to the bottom of the dish. This permitted spontaneous resealing of the lips of the cut, thus preventing direct access of oxygen to the submerged inoculum. When the lips of the cut did not reseal spontaneously, they were pressed together with the needle.

Inoculum was taken from 48-hour cultures on Sabouraud's glucose agar incubated at room temperature (25° to 28° C.).

Four parallel streaks, 2 for each of 2 isolates, were accommodated per plate, those of each isolate at a distance of approximately 1 cm. from each other, and in turn 3 cm. from those of the other isolate. Each isolate was inoculated into each of the 3 media being compared. The inoculated plates were incubated at uncontrolled room temperature (25° to 28° C.) in an inverted position, and examined, still inverted, directly under the low power of the microscope after 1, 2, 4, and 7 days postinoculation. When chlamydospores were not seen, mounts were prepared to permit a systematic search of small areas with the higher magnifications required for morphologic detail. Blocks of media, approximately 7 $mm.^3$ containing the running inoculated cut, were placed upon slides with a drop of lactophenol mounting fluid, a cover slip applied, and the preparation heated gently. With practice the agar could be melted without boiling, the fungous elements so preserved in their original positions.

Concurrently, the carbohydrate fermentation pattern was determined using a basal broth prepared according to Martin and his associates.[11] To this basal broth, before use, 0.5 ml. of a Seitz filtered 20 per cent aqueous solution of the carbohydrate to be tested was added. In this work, C.P. glucose, maltose, sucrose, and lactose were used. Inoculum was taken from 48-hour-old cultures on corn meal infusion agar plates. Inoculated carbohydrate broths, in Durham fermentation tubes, were incubated without sealing at room temperature and examined at the end of 2, 4, 7, and 14 days postinoculation for production of acid and gas.

RESULTS

This procedure enabled us to identify 113 or 63 per cent of 179 freshly isolated yeasts as *Candida albicans*. The remaining yeasts were identified as

*Chlamydospore Agar, Difco Laboratories.

(1) *C. parapsilosis,* 44 isolates or 24.5 per cent; (2) *C. tropicalis,* 5 isolates or 2.8 per cent; (3) *C. guilliermondii,* 3 isolates or 1.6 per cent; and (4) non-filamentous, asporogenous yeasts, 14 isolates or 8 per cent.

Of the 113 isolates of *C. albicans,* 109 were identified by their production of chlamydospores in at least 1 of the 3 media. The percentage of positive identification after 24 hours was 29.3 per cent on Chlamydospore Agar, 49.3 per cent on unpurified soluble-starch agar, and 55.0 per cent on corn meal infusion agar; after 48 hours increasing to 68.7, 68.0, and 85.3 per cent, respectively (Fig. 1). The incidence of chlamydospores increased at 4 days to 87.1 per cent on Chlamydospore Agar, 70.6 per cent on unpurified soluble-starch agar, and 94.4 per cent on corn meal infusion agar; final observation at the seventh day yielded percentages of 90.9, 72.6, and 98.2 per cent, respectively.

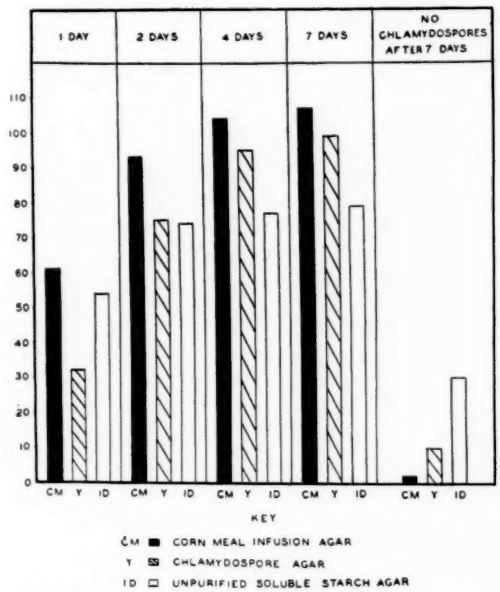

Fig. 1.—The production of chlamydospores by 109 isolates of *Candida albicans* on 3 media.

In 8 isolates diagnosis rested on the appearance of chlamydospores on one medium only: one producing them only on Chlamydospore Agar; 7 only on corn meal infusion agar. Three isolates failing to produce chlamydospores on Chlamydospore Agar did so on both corn meal infusion agar and unpurified soluble-starch agar; 1 isolate failing on corn meal infusion agar was positive on both Chlamydospore Agar and unpurified soluble-starch agar. Twenty-two isolates failing in the same manner on unpurified soluble-starch agar produced chlamydospores on both Chlamydospore Agar and corn meal infusion agar.

Four isolates, not included in these tabulations, had failed to produce chlamydospores on any medium during the 7-day observation period. When

retransferred to fresh corn meal infusion agar, 2 isolates produced chlamydospores within 24 hours. The remaining achlamydosporous isolates were identified as *C. albicans* on the basis of their microscopic morphology, carbohydrate fermentations, rabbit pathogenicity according to Benham,[3] and carbon assimilations modified after Wickerham.[17]

Fermentation reactions are defined here as the production of acid or acid and gas in the previously mentioned carbohydrate broths. Visibly detectable acid and gas were produced by 95.6 per cent of the 113 isolates on both glucose and maltose within 7 days, and by 99.1 per cent within 14 days. Acid was produced by 61.0 per cent from sucrose within 14 days; no strain, however, produced visibly detectable gas on sucrose within the same period. Further, on lactose, there was no visible production of acid and gas by any isolate.

DISCUSSION

For induction of chlamydospores in *C. albicans,* corn meal infusion agar, prepared and inoculated as described above, was superior to Chlamydospore Agar and unpurified soluble-starch agar. Not only did corn meal infusion agar induce chlamydospores in a higher percentage of isolates, but it did so in a shorter period of time.

The same medium also proved useful for the rapid morphologic identification of *C. parapsilosis, C. tropicalis,* and *C. guilliermondii.*

Even though Chlamydospore Agar is of "known chemical composition" and is available commercially, and soluble-starch agar is a simpler medium to prepare and more transparent than corn meal infusion agar, it is clear that corn meal agar continues to be the medium of choice for diagnosis of *Candida albicans.*

Since the preparation of corn meal infusion agar requires the use of a complex natural product and a prolonged period of extraction, investigators continue to try to simplify the preparation of this medium. A rapid method of extraction by boiling corn meal for 5 minutes has been used by Bakerspigel.[2] A similar rapid method of extraction has been tried by Wheeler and Little.[16] Comparison of the chlamydospore-inducing ability of these rapidly extracted corn meals with the slower method recommended by Benham,[3] and also in this paper, should be conducted before the quick-extract media can be safely used.

A more rational approach would be to continue attempts to identify what in the corn meal infusion is responsible for the induction of chlamydospores; this is being attempted in our laboratory.

Other species of the genus Candida are also known to produce chlamydospores. These are *C. tropicalis* and *C. stellatoidea.* The chlamydospores of *C. tropicalis* are, however, rarely produced, scant and atypical in morphology, and their appearance in isolates with the microscopic morphology and other diagnostic characteristics of *C. tropicalis*[3] is a reasonable safeguard against the possible misdiagnosis of this species. The chlamydospores of the so-called *C. stellatoidea,* on the other hand, are indistinguishable from those of *C. albicans.* The

validity of *C. stellatoidea* as a species, however, has been seriously questioned,[10, 18] and this organism is now considered merely an avirulent variant of *C. albicans.*

SUMMARY

The morphology and conditions known to favor the induction of the chlamydospores of *Candida albicans* are described.

Three media are compared for their chlamydospore-inducing ability with 109 freshly isolated strains of *C. albicans.* Corn meal infusion agar was found superior to Chlamydospore Agar (Difco) and an unpurified soluble-starch agar (experimental), both as to number of isolates induced to produce chlamydospores and as to the rapidity of the induction.

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A STABLE IRON REAGENT FOR DETERMINATION OF CHOLESTEROL

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INTRODUCTION

THE iron reagent introduced by Zlatkis and co-workers[1] and MacIntyre and Ralston[2] is a sensitive and reliable reagent for the estimation of cholesterol in biologic fluids. Although Zlatkis et al.[1] states that the reagent is stable for weeks, Zak and his associates[3] indicate that the reagent has limited stability. We have found that the stock ferric chloride solution, in glacial acetic acid, develops a precipitate of ferric oxide unless the solution is kept frozen. We have also found that the color reagent, prepared by mixing stock iron solution with sulfuric acid, develops a precipitate within a few days. It occurred to us that the iron reagents could be stabilized by dissolving the ferric chloride in phosphoric acid instead of acetic acid. The iron stock solution, in phosphoric acid, was found to remain stable indefinitely at room temperature. A color reagent containing phosphoric acid was developed which remained clear for 6 to 8 weeks and our findings form the basis for this report.

MATERIALS AND METHODS

The absorption spectrum of the purple cholesterol-iron complex obtained with a Beckman D U spectrophotometer, shows a sharp absorbing maximum at 560 mμ.[1] The purple color of the cholesterol-iron complex follows Beer's law to an absorbance of 1.0 with the Sheard Sanford photometer. Other photometers are equally suitable.

Reagents.—

Iron stock solution: Dissolve 2.5 Gm. $FeCl_3 \cdot 6H_2O$ in 100 ml. of phosphoric acid (87 per cent).

Color reagent: Dilute 8.0 ml. of iron stock solution to 100 ml. with concentrated sulfuric acid (98 per cent).

Effect of Acid Concentration on Color Production.—The formation of color depends on the heat of reaction produced when sulfuric and acetic acids are mixed. The proportion of acids necessary for maximum color formation was determined by varying the ratio of sulfuric and acetic acids. The final volume, iron content, and cholesterol concentrations were maintained at optimum conditions as described under procedure. The optimum ratio of

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Received for publication Jan. 24, 1957.

sulfuric to acetic acid ranged from 0.70 to 0.80 (Fig. 1). An acid ratio of 0.75 was selected, therefore, for the reaction. This ratio is at variance with the ratio of 0.65 used by other investigators.[1-3]

Effect of Iron Concentration on Color Production.—The concentration of ferric iron necessary for maximum color production was determined with color reagents containing either acetic or phosphoric acids. The concentrations of acetic or phosphoric acids were selected so that the optimal acid ratios for color formation were obtained. The various color reagents were added to cholesterol dissolved in 5 ml. of acetic acid as given in the procedure.

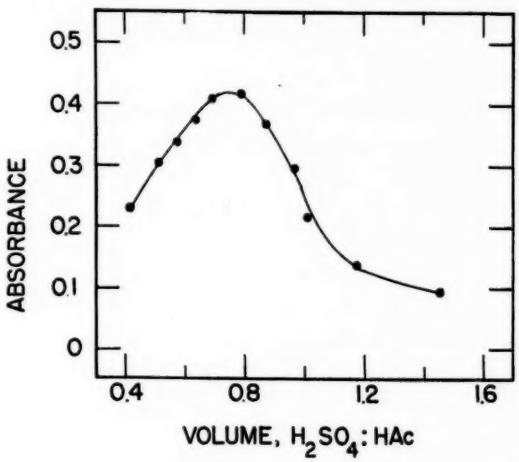

Fig. 1.—The ratio of sulfuric acid to acetic acid versus absorbance of color formed. All reactions were performed in a final volume of 9 ml.

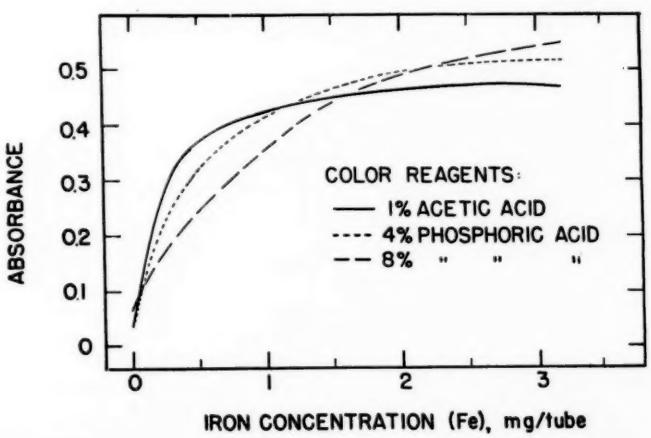

Fig. 2.—The concentration of iron versus absorbance of color formed, in the presence of varying amounts of acetic acid and phosphoric acid.

The formation of color increases rapidly as the concentration of iron increases (Fig. 2). With the color reagent containing acetic acid, very little additional color is formed at high concentrations of iron. The substitution of phosphoric acid for acetic acid in the color reagent decreases the availability

of iron by complex formation, and the curves no longer show any definite plateau region. The bright yellow color of the ferric phosphate solutions have negligible absorbance at 560 mμ.

Stability of Color Reagents.—The yellow stock iron reagent in phosphoric acid is stable indefinitely at room temperature. Iron reagents in sulfuric acid containing 1 per cent acetic acid, as prepared by previous workers,[1-3] develop a precipitate of ferric sulfate within a week after preparation. If the color reagent is prepared to contain 1 per cent phosphoric acid, the solution remains clear for about 3 weeks. Color reagents prepared to contain 4 to 8 per cent phosphoric acid remain clear for as long as 6 to 8 weeks, respectively, if protected from atmospheric moisture by a drying tube of silica gel, or when stored in glass stoppered containers open to the atmosphere only for the withdrawal of solution. Since the sensitivity of the color reagent depends on the heat produced on mixing sulfuric and acetic acids, inclusion of more than 8 per cent phosphoric acid to stabilize the iron results in less heat and less color formation. During this time, however, the bright yellow color of the ferric phosphate gradually fades as complex iron salts are formed. The formation of complex salts gradually reduces the concentration of available iron and decreases color production. The decrease in color as the solution ages is approximately 2 per cent weekly. However, the reagent is usable until a definite precipitate develops.

Time for Color Formation.—Maximum color formation is obtained 30 minutes after mixing the reagents but the color may remain constant for an additional 30 minutes. In humid atmospheres, however, the color begins to fade at a rate of 1 to 5 per cent per hour as the strong acid solution takes up water from the air. For this reason, all absorbancies were determined 30 minutes after mixing.

Procedure.—The extraction of cholesterol and its esters from biologic fluids and the separation of free cholesterol are not described in this report since adequate techniques are available in the literature.[2, 4] With the above conditions of the reaction defined, the following procedure was adopted.

To a suitable flask or tube containing 40 to 180 mcg. dry cholesterol, or its equivalent digitonide, add 5 ml. glacial acetic acid and mix well to dissolve the dry residue. Warming the acetic acid to 70° C. may aid dissolution of the material. Add 4 ml. of color reagent to the cooled acetic acid solution (room temperature) and mix gently, but thoroughly. Avoid vigorous shaking which produces annoying bubbles. A reagent blank and a standard amount of cholesterol should be carried through the procedure with each series of unknown samples. Determine the absorbance 30 minutes after mixing and compare with the standard amount of cholesterol after appropriate corrections for the blank solution.

RESULTS AND DISCUSSION

It is well known that the Lieberman-Burchard reaction, in chloroform solution, yields more color with esterified cholesterol than with free cholesterol, although Carr and Drekter[5] indicate that the chromogenicity of the Lieberman-Burchard reagent in acetic acid solvents is the same for both forms of cholesterol. The chromogenicity of the iron reagent for free and

esterified cholesterol determined on samples extracted with acetone:alcohol by the procedure of Zak and co-workers[3] and compared with similar samples of serum saponified and extracted by the procedure of Abell and associates[6] indicates the same color production for all forms of serum cholesterol (Table I), confirming previous reports and eliminating the need for saponification. Although total cholesterol may be determined directly on serum dissolved in glacial acetic acid[1, 3] thus eliminating any extraction procedure, we prefer to extract serum since the ratio of free cholesterol to esterified cholesterol is of importance in disease states. Extraction of cholesterol from serum also decreases the possibility of analytical error due to the presence of protein associated pigments in sera that have absorbancies similar to the color formed in the reaction. The use of serum directly may also involve an error due to the reaction of tryptophane with glyoxylic acid which may be present in some bottles of glacial acetic acid.[3] Since an extraction procedure eliminates most of the tryptophane, redistilled acetic acid is not necessary and commercially available reagent grade material may be used.

TABLE I. COMPARISON OF DIRECT ANALYSIS AND SAPONIFICATION OF CHOLESTEROL IN SERUM EXTRACTS WITH THE IRON REAGENT

SERUM	DIRECT* (MG./100 ML.)	SAPONIFICATION† (MG./100 ML.)
1	136	136
2	163	162
3	213	213

*Method of Zak et al.[3]
†Method of Abell et al.[6]

TABLE II. RECOVERY OF CHOLESTEROL ADDED TO SERUM WITH THE IRON REAGENT

SAMPLE*	NO. OF DETER.	CHOLESTEROL ADDED (MG./100 ML.)	CHOLESTEROL RECOVERED (PER CENT)
Total cholesterol	15	100	100 ± 3.0†
	17	200	100 ± 3.0
	7	300	102 ± 2.6
Free cholesterol	15	25	103 ± 4.1
	12	50	98 ± 5.7
	8	100	97 ± 7.9

*Samples prepared by the method of Zak et al.[3]
†S.D. = $(\Sigma d^2/n)^{1/2}$.

TABLE III. COMPARISON OF IRON REAGENT AND LIEBERMAN-BURCHARD REACTION FOR SERUM CHOLESTEROL

SAMPLE*	NO. OF ASSAYS	IRON REAGENT (MG./100 ML.)	LIEBERMAN-BURCHARD (MG./100 ML.)
Total cholesterol	15	217 ± 7.0†	216 ± 8.9†
Free cholesterol	8	47 ± 3.7	49 ± 4.3

*Samples prepared by the method of Sperry and Webb.[4]
†S.E. = $[\Sigma d^2/n(n-1)]^{1/2}$.

Recovery studies, performed by adding known amounts of cholesterol to serum, followed by extraction and analysis by the procedure of Zak et al.[3] using the new iron reagent described in this report were satisfactory as shown

in Table II. A comparison study of the new iron reagent and the Lieberman-Burchard reaction on serum samples prepared by the procedure of Sperry and Webb,[4] also show satisfactory agreement (Table III).

SUMMARY

A new, stable iron reagent that yields results comparable to other procedures and eliminates the need for preparing color reagents at more than infrequent intervals is described.

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NEW, RAPID, AND PRACTICAL MICROMETHODS FOR PROTHROMBIN DETERMINATION

I. The Watch Glass Method

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THE introduction of the one-stage prothrombin-time test by Quick initiated the remarkable advances in the study of blood coagulation and its abnormalities over the last 15 years. The test is widely used today as the best test for the control of anticoagulant therapy and is as well an important tool in coagulation research studies.

While possessing many merits, the test does have certain disadvantages. The proper performance of the test usually requires that the patient be in the hospital, have accessible veins for puncture, and that the test itself be carried out under relatively standard conditions in the hospital laboratory.

It would be advantageous for the physician to have this very useful test available at the bedside, in the operating room, or in his private office. Further, the avoidance of repeated venipuncture and the practical possibility of an immediate result would make the test even of greater value both clinically and experimentally. The one-stage test as performed today does not include these desirable features. Consequently, we have modified the Quick prothrombin-time test to produce, in effect, two micro tests for obtaining accurate and immediate results utilizing one or two drops of blood obtained by simple finger stick or pick up from oozing or injured surfaces.

It is the purpose of this paper to describe one of the two tests and to report the results obtained with it on over 100 patients receiving anticoagulant therapy, as well as to compare the results with simultaneously obtained Quick one-stage values. Another micromethod, the so-called "Hematocrit method" is reported elsewhere in this Journal.

MATERIALS

White blood cell pipettes of the usual type were employed.

Watch glasses, glass diameter = 6.5 cm. were used. These were acid washed and not siliconized.

Thromboplastin, prepared as previously described.[1]

Stop watch, an ordinary hand stop watch was employed.

Lancets were used for finger stick.

METHODS

Performance of the Test.—(Fig. 1.) Thromboplastin is drawn to the "1" mark of a white blood cell counting pipette and placed in a watch glass. An equal amount of blood

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Received for publication Jan. 24, 1957.

is drawn with a second pipette from a freely flowing finger stick and blown into the thromboplastin, and immediately a stop watch is started. Mixing of the two may be enhanced by gently blowing through the pipette for a few seconds. After a pause of ten seconds, the watch glass is tilted every second and observed over a white paper or a flashlight until fibrin strands or gel formation sets in. This is a clear-cut end point. The room temperature is noted.

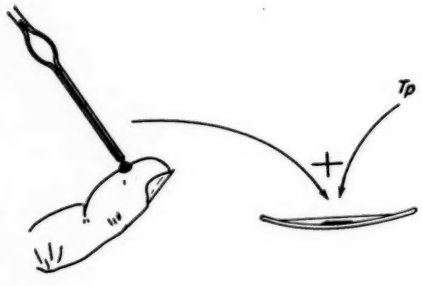

Fig. 1.

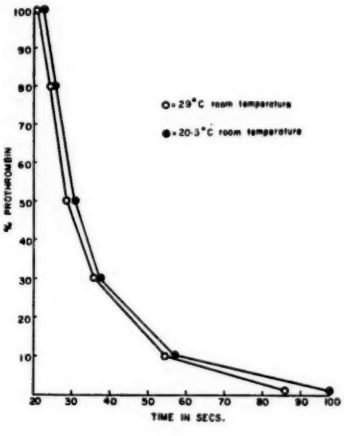

Fig. 2.

Fig. 1.—The essential steps in the performance of the Watch Glass test are illustrated. After simple finger stick with a Hemolet, the first few drops of blood are wiped away and blood is drawn to the 1 mark of the white blood cell pipette. This is quickly mixed with an equal amount of thromboplastin already contained in the watch glass. At the time of mixture, a stop watch is started. The end point is clear-cut. The room temperature is noted.

Fig. 2.—Dilution curves, Watch Glass method. Shown is the dilution curve used to convert clotting times to "per cent prothrombin." This is a function of the room temperature, the thromboplastin preparation, and the blood sample. Values may be interpolated for room temperatures in between those shown in the figure.

Conversion of Clotting Time to "Per Cent Prothrombin."—A standard dilution curve similar to that of Quick was plotted by determining the clotting times of whole bloods of varying "per cent prothrombin" which were artificially prepared. This latter was simply accomplished in two steps: first, obtaining various "per cent prothrombin plasmas" by mixing aluminum-absorbed plasma (so-called prothrombin free plasma) and normal plasma in various proportions. Then, by mixing such plasmas with their well-washed cellular components in the proportion indicated by their hematocrit values, varying per cent prothrombin whole bloods were prepared. The standard dilution curves obtained at two extremes of room temperature are shown in Fig. 2.

RESULTS

In Fig. 3 is seen a comparison of results obtained on blood from over 100 patients, both normal and receiving anticoagulant therapy, with the Quick test and the Watch Glass test. The values are distributed around the theoretical regression line, $y = ax + b$ where $a = 1.00$ and $b = 0$. The least squares regression formula for the observed values is $Y = 1.019\ X + 0.16$. P value in a test of significance for linear regression of observed versus theoretical is greater than 0.9. The standard deviation of the Quick one-stage method in our laboratory was found to be 0.3 second at the 100 per cent prothrombin

level. This is equivalent to 6 per cent "prothrombin." The standard deviation of the Watch Glass method in our laboratory was 0.9 second. This is equivalent to 4.5 per cent "prothrombin."

DISCUSSION

The data obtained comparing the Watch Glass method with the Quick method for "prothrombin" determination on 108 patients are in good agreement with the theoretical values. The variations are quite comparable and the relative errors are also comparable. Thus the data are judged acceptable on a statistical basis. From a practical viewpoint, the test has proved quite helpful when anticoagulant therapy was needed on a patient whose veins were not palpable. Moreover, its usefulness in pediatric cases or where repeated sampling is needed seems evident.

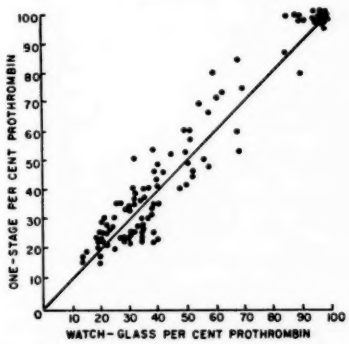

Fig. 3.—Correlation of prothrombin determinations. The scattergram represents the results obtained with 108 patients, both normal and receiving anticoagulant therapy. Prothrombin values by the Quick method and the Watch Glass method are plotted. The line of perfect correlation is indicated. The values obtained by both methods are statistically comparable.

Finally, assuming suitable controls are employed and a stable thromboplastic preparation is available, the method is adaptable to a bedside procedure, an office procedure, a pediatric procedure, or a procedure on patients without accessible veins.

SUMMARY

A new micromethod for prothrombin determinations is described. Results on over 100 patients with the Quick test and the Watch Glass method are presented. The usefulness of the test is discussed.

We wish to thank Miss Joyce Black for technical assistance.

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NEW, RAPID, AND PRACTICAL MICROMETHODS FOR PROTHROMBIN DETERMINATION

II. The Hematocrit Method

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THE need for micromethods of prothrombin estimation has been variously met in the past. Quick,[1] in 1939, reported a very simple bedside technique employing a drop of capillary blood and a drop of thromboplastin on a slide; he mixed and then watched for a suitable end point. (In this paper he criticized Smith and his associates for their proposed formula.) In 1940, Kato[2] employed capillary blood, oxalated it upon drawing, and thus required in the test the addition of calcium. His method correlated well with the Quick method; however, the procedure is long, very exacting, and requires considerable equipment for a "rapid" bedside technique. In 1941, Innes and Davidson[3] collected capillary blood in white blood cell counting pipettes, oxalated the blood in a watch glass, and then mixed it with snake venom. In 1942, Ulin and Barrows[4] described a new method employing a wire loop coagulomotor and capillary blood. Lufkin and Strolber,[5] using calibrated pipettes, delivered blood and thromboplastin in proportion of 1:4 into a watch glass. These last three methods all expressed results using the formula of Smith and associates. However, none of these methods has been widely accepted.

In the course of work, our laboratory was requested to contrive some manner of following anticoagulant therapy on a patient with no accessible surface veins. Further, the Watch Glass method[6] had not yet been adequately investigated and standardized. Such a method was devised, utilizing no principles other than those of the Quick one-stage procedure, save that only a few drops of whole blood from a finger stick served as the sample. It is the purpose of this paper to describe the method, the results obtained therewith, and its use as a special, though not difficult, laboratory test.

MATERIALS

Wintrobe hematocrit tube
Pasteur pipettes with long stem
Alumina-adsorbed plasma[8]
Thromboplastin (prepared as previously described[7])
Stop watch
Lancets
Test tubes, water bath[6]
Silicone, D.C. 200* was used as a 2 per cent suspension in CCl_4
Glassware so treated was dried in hot-air oven at 190° C. for 1 to 2 hours.

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Received for publication Jan. 24, 1957.

*Kindly supplied by the Hollingshead Company, Camden, New Jersey.

METHODS

Performance of the Test.—(Fig. 1.) The essential principle in this method is that the test sample of whole blood from a finger stick is received into a siliconized Wintrobe hematocrit tube which contains a known amount of "deprothrombinized" (alumina-adsorbed) plasma which is slightly overoxalated. After centrifugation, the additional test plasma volume may be simply calculated by reading the increase in total volume and subtracting the red cell volume at the bottom of the tube. Therefore, if the test sample contained 100 per cent "prothrombin," the plasma mixtures should give a "per cent prothrombin" value in a one-stage test equal to the per cent test plasma. Values of per cent prothrombin in the test plasma may be calculated by the formula:

$$\frac{\text{Per Cent Prothrombin Values of Mixture*}}{\text{Per Cent Test Plasma in Mixture†}} \times 100 = \text{"Per Cent Prothrombin in Test Plasma"}$$

It will be immediately recognized that this is the procedure for making prothrombin clotting time calibration curves and thus employs no new principles in the one-stage test. (Fig. 2.) A typical detailed procedure follows: 4.5 ml. normal human blood was collected in a test tube containing 0.5 ml. of 0.1 M oxalate. The plasma obtained by centrifugation was "deprothrombinized" by alumina gel adsorption. Its clotting time after adsorption

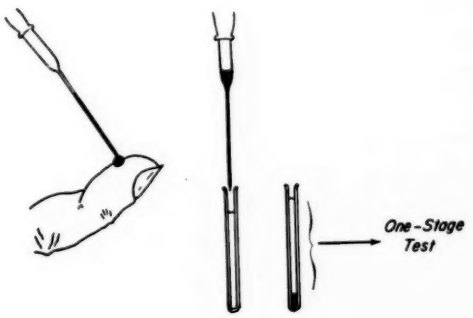

Fig. 1.

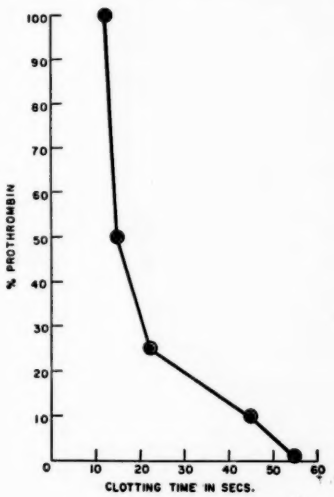

Fig. 2.

Fig. 1.—This figure illustrates the essential steps in the performance of the Hematocrit method for "prothrombin" estimation. After simple finger stick with a Hemolet, the first few drops of blood are wiped away, and blood is drawn into a siliconized Pasteur pipette. A convenient amount is then added to a Wintrobe hematocrit tube containing a known amount of slightly overoxalated, "prothrombin-free" plasma and mixed well. After centrifugation, the total volume and red cell volume are noted. The supernate plasma is then subjected to a regular Quick (prothrombin time) test. Calculation of per cent prothrombin in the patient's blood sample is described in the text.

Fig. 2.—One-stage curve. Shown is the dilution curve used to convert the clotting times of the plasma mixture to "per cent prothrombin." Calculation of the per cent prothrombin in the patient's blood sample is described in the text.

was more than four minutes. Exactly 0.50 ml. of the alumina-adsorbed plasma was placed in a clean siliconized Wintrobe hematocrit tube with a long-stemmed Pasteur pipette after 0.02 ml. of 0.1 M oxalate was added. The volume was then precisely read and noted to be 0.52. A few drops of blood from a clean finger stick were taken up in a siliconized long-stemmed Pasteur pipette, placed in the Wintrobe tube, and carefully and intimately mixed with the alumina plasma sample. After centrifugation, the total volume was read and noted to be 0.75 ml. The red cell volume was read and noted to be 0.10 ml. The additional test plasma was then calculated to be 0.13 ml. and present in the plasma mixture

*Obtained by converting clotting times of mixture to per cent prothrombin.
†Obtained by noting volumes in the hematocrit tube.

as 23 per cent (0.13 + 0.2/0.65). The clotting times obtained were 36.8 and 37.5 seconds. When the plasma mixture was analyzed by the conventional one-stage method, this was read to be equal to 15.5 per cent. Thus, the per cent prothrombin in the test plasma was calculated to be: $\frac{15.5}{23} \times 100 = 67$ per cent.

RESULTS

In Fig. 3 is seen a comparison of results obtained on blood from over 100 patients, both normal and receiving anticoagulant therapy with the Quick test and the Hematocrit test.

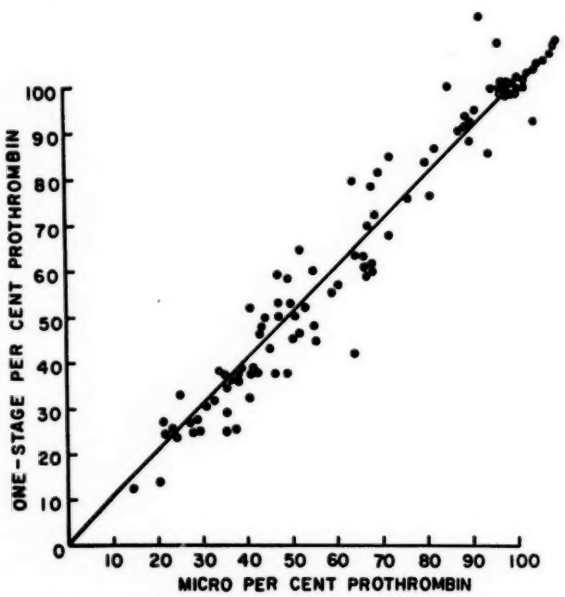

Fig. 3.—Correlation of prothrombin determinations. Shown in the scattergram are the results obtained with 108 patients, both normal and receiving anticoagulant therapy. Prothrombin values by the Quick method and the Hematocrit method are plotted. The line of perfect correlation is indicated. The values obtained by both methods are statistically comparable.

The values are distributed around the theoretical regression line, $y = ax + b$ where $a = 1.00$ and $b = 0.00$. The least squares regression formula for the observed values is $Y = 0.9646\ X + 0.37$. P value in a test of significance for linear regression of observed versus theoretical is greater than 0.9. Standard deviation = 8 per cent. The standard deviation of the Quick one-stage method in our laboratory was found to be 6 per cent. The standard deviation of the Hematocrit method in our laboratory was thus comparable.

DISCUSSION

The data obtained comparing the Hematocrit method with the Quick method for "prothrombin" determination on 108 patients are in good agreement with the theoretical values. The variations are quite comparable and the relative errors are also comparable. Thus, statistically, the data are judged acceptable.

A word should be said in interpretation of these results compared to the interpretation of the regular Quick one-stage results. It is widely accepted that the one-stage test is an integrated measure of the nonthromboplastic components of the coagulation mechanism. However, a normal clotting time in this micro one-stage test indicates a gross adequacy of only those components which were removed by the alumina adsorption: prothrombin and pro-SPCA (serum prothrombin conversion accelerator). Thus, this particular modification of the one-stage test, employing as it does an alumina plasma menstruum, may be said to differ from the regular one-stage test in that it provides a measure of prothrombin and pro-SPCA only.

SUMMARY

This test is practical and has proved helpful in certain clinical cases. Like the Watch Glass test, its value in patients "without veins," in pediatrics, or repeated samplings seems obvious. Further, its use in experimental procedures, for example, in small animal experimentation, would also seem advantageous.

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A METHOD FOR ESTIMATING THE BACTERIAL POPULATION OF THE OROPHARYNX

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IT IS difficult to quantify the bacterial flora of the oropharynx. This difficulty was encountered by us in a study which involved the daily variation of the bacterial population of the throat. The methods commonly used provide only a rough quantitation of the oropharyngeal bacteria.[1-6] Since the accuracy of the procedures in use have not been established, certain technical refinements seem essential in order to insure a more reliable analysis of the bacterial population of the throat.

The actual number and type of bacteria in the pharynx and their precise in vivo relationships to each other probably cannot be determined. For example, one cannot estimate the actual number of streptococci present in the oropharynx since the total quantity of pharyngeal secretions from which the counted aliquot is obtained cannot be determined. In addition, the growth of the various species of bacteria in vitro depends upon the medium used, the period of incubation, the O_2 and CO_2 tensions, and other environmental conditions.

The present investigation was limited to an evaluation of the aerobic streptococci and *Staphylococcus albus* in relation to other bacteria present in the oropharynx and as this is reflected on the specific cultural environment we used. A statistical analysis was then applied to determine the limits of accuracy of the technical procedures.

MATERIALS AND METHODS

A platinum inoculating loop was sterilized, cooled, and then rubbed across the surface of the oropharynx with care to avoid the uvula and tongue. The loop was then agitated in 1 ml. of sterile physiologic saline. The saline was immediately poured onto the surface of a 5 per cent sheep blood agar plate. Trypticase-soy agar was the basal medium. The plate was rotated to distribute the solution evenly over the surface and then incubated for 48 hours at 37° C.

Colony counts were made in the following manner: a section of the plate was arbitrarily selected and 200 or more of all types of colonies were counted and grossly identified. Counting was done with the aid of a hand lens. Verification of the species was accomplished by Gram stain and subculture to appropriate media when necessary. The percentage of each species was then calculated.

Two methods were used to test the reliability of the procedure. First, the wire loop was immersed in the same 2 ml. of saline after each of two swabbings of the patient's oropharynx. The sample was divided into two parts and each ml. sample was then plated.

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This investigation was supported in part by a Special Research Fellowship from the National Institute of Mental Health, Public Health Service.

Received for publication Feb. 25, 1957.

The two plates were treated as described above. Twenty healthy persons (hospital personnel) were tested in groups of 3 and 4 at a time. The plates were coded by a person other than the technician who did the counting in order to conceal the identity of the pairs.

In the second experiment, two consecutive cultures were taken from each person. The initial swabbing was introduced in 1 ml. of saline. The loop was sterilized over a flame, allowed to cool and the second swabbing was carried out in a similar manner and introduced into another ml. of saline. Each sample was cultured as described above. Approximately 2 to 3 minutes elapsed between taking the two independent, consecutive samples. These plates were also coded. Nineteen of the subjects used in the previous experiment were tested in this manner.

RESULTS

Alpha and gamma streptococci and *Staphylococcus albus* percentages were obtained. The results of the experiments are shown in Table I.

Single Sample Divided.—When the single samples were divided into two parts, the ninety-five per cent confidence statements, as determined by an analysis of variance technique, were found to be $x - 3.2 \leq \xi \leq x + 3.2$ for gamma streptococci and $x - 3.8 \leq \xi \leq x + 3.8$ for alpha streptococci, where x is observed per cent count and ξ is the "true" per cent, and where the distribution for per cent counts is assumed to be approximately of Gaussian form. When the alpha and gamma are added to obtain a total streptococcal count and then analyzed in a similar manner the ninety-five per cent confidence statement is $x - 3.9 \leq \xi \leq x + 3.9$. These values are in terms of per cent of the species of bacteria of the total of over 200 colonies of all species counted on the plate. The *Staphylococcus albus* counts are also shown in Table I; however, the large number of samples with no growth of this organism precludes the assumption of Gaussian distribution.

Two Consecutive Samples.—The ninety-five per cent confidence statements in the experiment where two independent samplings of the oropharynx were obtained at a 2- to 3-minute interval of time were $x - 2.9 \leq \xi \leq x + 2.9$ for gamma streptococci and $x - 5.2 \leq \xi \leq x + 5.2$ for alpha streptococci. For total streptococci the confidence statement was $x - 3.5 \leq \xi \leq x + 3.5$. For the reason mentioned in the previous paragraph, no confidence limits were determined for *Staphylococcus albus* counts. They are, however, listed in Table I.

Beta hemolytic streptococci were not isolated in any of the cultures, possibly because the cultures were obtained during the summer months. Other bacteria isolated included Neisseria (chiefly *N. catarrhalis*), diphtheroids, *Staphylococcus aureus, Hemophilus parainfluenzae,* and *Diplococcus pneumoniae.*

DISCUSSION

The first experiment, which involved dividing a single sample into two parts and culturing and counting each separately, provides limits of the reliability of the procedure after the sample has been taken from the oropharynx. These limits are roughly 3 plus per cent on either side of the true count for alpha and gamma and total streptococci. The second experiment which involved consecutive samples, taken at about 2-minute intervals, gives a further index of the added error in the swabbing procedure. This, however, would assume that no

TABLE I

SUBJECTS	ONE SAMPLE DIVIDED INTO TWO PARTS (PER CENT COUNTS)						DAYS BETWEEN EXPERIMENTS	TWO CONSECUTIVE SAMPLES (PER CENT COUNTS)					
	GAMMA STREPTOCOCCI		ALPHA STREPTOCOCCI		STAPHYLOCOCCUS ALBUS			GAMMA STREPTOCOCCI		ALPHA STREPTOCOCCI		STAPHYLOCOCCUS ALBUS	
1	6.9	7.0	62.5	61.8	4.9	5.1	23	1.4	0.9	83.8	78.6	1.4	1.8
2	12.3	11.0	52.6	52.6	0.9	0.0	23	3.9	4.8	65.4	66.7	0.5	0.0
3	10.5	13.6	4.1	6.6	0.0	0.0	21	21.5	21.5	38.5	35.4	1.4	0.6
4	92.4	92.2	5.6	5.4	0.0	0.0	21	57.1	56.5	25.7	25.6	0.3	0.3
5	52.5	51.2	39.6	35.5	1.3	1.2	79	38.8	36.0	53.6	50.8	0.9	1.1
6	80.5	79.5	18.0	18.6	0.0	0.6	75	15.5	16.0	24.1	25.3	0.5	0.4
7	85.5	85.1	13.2	13.6	0.3	0.3	16	30.6	29.9	56.9	59.2	1.2	1.9
8	27.0	26.8	45.6	46.3	2.1	1.6	16	47.1	47.6	39.0	38.1	0.7	2.4
9	19.0	23.0	66.7	64.7	1.6	1.4	15	14.9	19.3	59.8	69.1	0.4	0.0
10	3.2	2.3	66.0	73.4	0.0	0.0	15	5.2	7.9	64.8	61.1	1.1	0.9
11	21.0	22.5	64.8	60.7	2.5	0.5	15	16.5	15.9	53.6	54.2	0.3	0.3
12	34.2	27.9	53.9	55.7	0.7	0.4	70	15.2	17.9	70.1	66.1	0.3	0.0
13	4.1	5.6	52.2	55.2	4.1	5.0	70	30.0	29.8	45.0	46.5	0.5	0.0
14	35.2	37.4	21.4	20.9	0.0	0.5	0	39.3	35.4	17.0	22.0	0.0	0.0
15	14.0	13.3	50.8	48.1	0.0	0.4	0	14.4	15.6	51.7	50.6	0.3	0.3
16	65.3	66.9	19.5	19.1	0.0	0.0	0	61.6	61.3	20.5	21.5	0.0	0.8
17	28.7	30.3	33.1	33.8	0.8	0.3	0	25.7	26.5	39.3	38.1	1.5	0.9
18	10.8	10.8	28.1	26.6	0.0	0.0	0	13.8	10.5	21.3	27.0	0.0	0.0
19	46.4	44.1	42.2	42.6	0.0	0.0	15	22.2	23.1	53.0	52.4	1.3	1.1
20	21.0	22.5	49.5	46.1									
95 per cent confidence statement*	$x - 3.2 \leqslant \xi \leqslant x + 3.2$		$x - 3.8 \leqslant \xi \leqslant x + 3.8$					$x - 2.9 \leqslant \xi \leqslant x + 2.9$		$x - 5.2 \leqslant \xi \leqslant x + 5.2$			

*Where x is the observed per cent count and ξ is the "true" per cent count.

change occurred in the flora during the intervening 2 minutes. There was no difference in the limits of error for the divided single sample and the two consecutive samples for gamma streptococci. However, there was some increase in error in consecutive sampling for alpha streptococci. Total streptococci remained the same in both cases and essentially averaged out the gamma and alpha counts. In any event, it is apparent that estimates of these bacteria in the oropharynx can be made with some precision. There was not a significant regression of the magnitude of discrepancy between the two samples on the size of the count. This suggests that the estimated variance applies approximately to large or small percentage counts alike. All counts were done by the same technician, however, and do not preclude any consistent errors of identification she could have made.

We were interested in the variation of the counts when two swabs were taken independently but within a short span of time as was done in the second experiment. The results indicate that the flora do not vary greatly from moment to moment and that the two separate swabbings pick up essentially the same distribution of these bacteria. Some further observations can be made of changes that occur in the percentage counts over longer periods of time. This can be done by comparing the values obtained on each subject for both experiments. The same subjects were used for both experiments and the time that elapsed between the experiments varied. The interval was approximately 3 weeks for the first 4 subjects, 2½ months for the fifth and sixth, 2 weeks for Subjects 7 to 11, and 2 months for Subjects 12 and 13. Both experiments were done on the same day and within the same hour for Subjects 14 to 18 but there was a lapse of 2 weeks for Subject 19. The changes were minimal when both tests were done within the same hour (Subjects 14 to 18). Examples of both increase and decrease in percentage of these bacteria were found when the time interval extended from 2 weeks to 2½ months.

It is difficult to interpret the results of the *Staphylococcus albus* counts. They show some degree of reproducibility but the persistently low percentage range magnifies the differences between two samples. It would appear that any attempt to follow daily variations would be inaccurate unless the counts ranged into higher values.

Ordinarily, cotton swabs have been used to obtain samples from the oropharynx. The swabs are then streaked directly onto the surface of the medium. We found that this technique causes overgrowth and crowding of the colonies. In addition, the possibility of trapping the organisms in the cotton fibers exists. Some investigators have introduced the swab into broth and mixed by repeated pipetting of the broth.[3-5] We found that an ample number of organisms could be picked up on a platinum inoculating loop and although we could not be certain, we felt that there was a better possibility for transport of organisms into the solution. Practically all of the subjects felt that this procedure was less uncomfortable than sampling with a cotton swab. Flooding the resulting diluent across the surface of the medium provided good distribution of colony growth. It reduced crowding and provided good conditions for identification

and counting. One milliliter of diluent and 48-hour incubation provided optimal conditions for counting the bacterial colonies that were of particular interest to us. Sheep blood agar was superior to human blood agar for differentiation of hemolytic and nonhemolytic streptococci.

SUMMARY

Some refinements of the technique of estimating the streptococcal and staphylococcal population of the oropharynx are described. Statistical analysis of the data derived by the use of these refinements indicates that the oropharyngeal flora can be estimated in more precise terms than heretofore described.

We wish to acknowledge with gratitude the assistance of Malcom Turner, Biostatistics Department, Kettering Laboratory, and Dr. Dorothy Fleming, Department of Microbiology of the University of Cincinnati College of Medicine.

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